学位申請論文

小笠原道生

主論文要旨

内柱は尾索類、頭索類、円口類の幼生に存在する咽頭内器官である。内柱はまたヨードの集積機能およびヨードの代謝能を持つことから、脊椎動物の甲状腺の相同器官であると考えられている。脊椎動物の *TTF-1*（甲状腺転写因子 1）遺伝子および *TPO*（甲状腺パーオキシデース）遺伝子はその機能に関連して甲状腺で発現しており、転写因子 TTF-1 は甲状腺ホルモンの合成に関与するヨード化酵素 TPO の発現を制御している。一方、尾索類ホヤの *TTF-1* 遺伝子および *TPO* 遺伝子は内柱で特異的に発現しているが、これらの遺伝子の発現領域が重なり合わないことから、ホヤ *TPO* 遺伝子の発現は転写因子 TTF-1 によって制御されていると考えにくい。内柱の発生および機能発現の分子的なメカニズムを、特に甲状腺への進化を考慮に入れつつ明らかにするため、本研究では頭索類ナメクジウオの *Branchiostoma belcheri* から *TTF-1* 遺伝子（*BbTTF-1*）および *TPO* 遺伝子（*BbTPO*）の cDNA クローンを単離した。ナメクジウオの *TTF-1* 遺伝子および *TPO* 遺伝子は RT-PCR/サザンブロット解析により主に成体の内柱で発現していることが明らかになった。また *in situ* ハイブリダイゼーションによる時間的・空間的な発現パターンの解析から、*BbTTF-1* は初期胚では内胚葉細胞で発現し、成体の内柱のすべてのゾーンで発現が維持されることがわかった。一方、*BbTPO* は成体の内柱のゾーン 5 および 6 で強く発現し、さらにゾーン 1 および 3 でも弱い発現がみられ、*BbTTF-1* の発現と重なる。この両遺伝子の内柱での発現は、内柱が甲状腺と相同であることを強く示唆する。また、ナメクジウオにおけるこれらの遺伝子の時間的・空間的にオーバーラップした発現は、転写因子 TTF-1 が *TPO* 遺伝子の発現を制御する可能性を示唆し、さらに TTF-1 による *TPO* 遺伝子の発現の制御が頭索類と脊椎動物の共通祖先で獲得されたことを示唆する。

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Overlapping expression of amphioxus homologs of the thyroid transcription factor-1 gene and thyroid peroxidase gene in the endostyle: insight into evolution of the thyroid gland

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Abstract The endostyle is a pharyngeal organ of urochordates, cephalochordates and primitive vertebrates. This organ has iodine concentrating and iodine- metabolism activities, and therefore the endostyle is considered to be homologous to the follicle of the thyroid gland. In higher vertebrates, both the *TTF-1* (thyroid transcription factor-1) and *TPO* (thyroid peroxidase) genes are expressed in the thyroid gland follicle. TTF-1 regulates the expression of *TPO*, which encodes an iodinating enzyme associated with thyroid hormone synthesis. A recent study showed that the ascidian *TTF-1* and *TPO* genes were specifically expressed in the endostyle, but that the expression domains of these genes were not overlapping, suggesting that ascidian *TPO* is not regulated by TTF-1. To investigate the molecular mechanisms involved in the formation and function of the endostyle, with special reference to the evolution of the follicle of the thyroid gland, I isolated and characterized cDNA clones for the amphioxus homologs of the *TTF-1* gene (*BbTTF-1*) and *TPO* gene (*BbTPO*) from *Branchiostoma belcheri*. RT-PCR/Southern blotting revealed that both amphioxus *TTF-1* and *TPO* were mainly expressed in the adult endostyle. Spatial and temporal expression patterns assessed by in situ hybridization revealed that expression of *BbTTF-1* occurred in the endodermal cells during early embryogenesis, and was maintained in all zones of the adult endostyle. On the other hand, expression of *BbTPO* was chiefly in zones 5 and 6 of the adult endostyle where it overlapped with that of *BbTTF-1*, and to a lesser extent in zones 1 and 3. This restriction of the expression of *BbTTF-1* and *BbTPO* to the endostyle strongly suggests that the endostyle is homologous to the follicle of the thyroid gland. Moreover, the spatial and temporal expression patterns of these genes suggest that TTF-1 may regulate *TPO* expression. The co-expression of these genes in amphioxus suggests that regulation of *TPO* by TTF-1 was present in the common ancestor of cephalochordates (acraniates) and craniates.

Key words Amphioxus, TTF-1, TPO, Endostyle, Thyroid gland, Gene expression, Evolution

Introduction

The endostyle is located in the ventral part of the pharynx of urochordates (tunicates), cephalochordates (acraniates) and ammocoetes (larval lampreys). In addition to the notochord, dorsal hollow neural tube and pharyngeal gill slit, the endostyle is, therefore, a key structure for understanding the origin and evolution of the chordate body plan (e.g., Brusca and Brusca 1990; Willmer 1990; Nielsen 1995; Gee 1996).

The chordate endostyle is thought to have two major functions. One is in protein-secretion for catching food particles from sea water, i.e., for filter feeding. Ultrastructural studies of the endostyle demonstrated that the endostyle contains several types of glandular cells, which occupy a large part of the endostyle (Barrington 1958; Fujita and Honma 1968; Fujita and Nanba 1971). Previous molecular studies of ascidians endostyle indicated that large amounts of transcripts which encode the predicted secretory proteins were expressed in zones of the protein- secreting elements (Ogasawara et al. 1996; Ogasawara and Satoh 1998). The other function of the endostyle appears to be equivalent to that of the vertebrate thyroid gland follicle. Histochemical studies in the ascidian (Thorpe et al. 1972; Dunn 1974; Fujita and Sawano 1979), amphioxus (Tsuneki et al. 1983) and larval lamprey (Fujita and Honma 1968) revealed iodine concentrating and thyroid peroxidase activity in the endostyle (reviewed by Eales 1997). Because of these thyroid-like properties and because the lamprey endostyle transforms to the follicle of the thyroid gland during the metamorphosis (Wright and Youson 1976), the endostyle is generally accepted to be homologous to the vertebrate follicle thyroid gland and to be its evolutionary precursor (e.g., Barrington 1957; Salvatore 1969; Thorpe et al. 1972; Dunn 1974, 1980; Fujita and Sawano 1979).

In the adult amphioxus, the endostyle forms a groove in the floor of the pharynx and extends from the forepart of the pharynx to the esophagus (see Fig. 6E). Morphological observations of the amphioxus endostyle demonstrated that this organ forms a trough-shaped structure similar to the ascidian endostyle and that the organ is divided into six different zones that run parallel to one another in longitudinal orientation (Barrigton 1958). Ultrastructural and histochemical studies of the amphioxus endostyle revealed that histochemical activities of peroxidase (Tsuneki et al. 1983; Fredriksson et al. 1985) and iodine-concentrating activity (Thorpe et al. 1972; Dunn 1974; Fredriksson et al. 1984; Ericson et al. 1985; Fujita and Honma 1969) were detected in the cells of zones 5 and 6, and therefore these zones are considered to be the thyroid-equivalent region.

The thyroid gland follicle of higher vertebrates concentrates iodine and synthesizes thyroid hormones. Several thyroid-specific molecules have been isolated which are essential for thyroid hormone synthesis. These include Tg (thyroglobulin), an iodinated protein (Malthiery and Lissitzky 1987; Caturegli et al. 1997; Di Lauro et al. 1985; Mercken et al. 1985; van Ommen et al. 1989) and TPO (thyroid peroxidase), which iodinates Tg (Taurog 1974; Fujita and Sawano 1979; Kimura et al. 1989; Kotani et al. 1993; Isozaki et al. 1989; Magnusson et al. 1987). Both occur in the differentiated thyroid follicle, and are therefore useful markers of the vertebrate thyroid gland follicle. In addition, several transcription factors have been identified (Lazzaro et al. 1991; Plachov et al. 1990; Zannini et al. 1997), including *TTF-1* (also known as *Nkx-2.1*, *Titf1*, and *T/ebp*) which encodes a protein containing an NK-2 type homeodomain. Vertebrate *TTF-1* is expressed in the follicular thyroid gland, in the lung and in the anterior nerve cord (Lazzaro et al. 1991; Civitareale et al. 1993; Kimura et al. 1996, Pera and Kessel 1998). In the follicular thyroid gland, TTF-1 directly regulates expression of *Tg*, *TPO* and the thyrotropin receptor gene (Damante et al. 1994; Civitareale et al. 1993; Mascia et al. 1997). The *TTF-1* gene has also been isolated from amphioxus, where it is expressed in the larval endostyle and other tissues; however expression in adults was not determined (Venkatesh et al. 1999).

We have isolated and characterized several endostyle-specific genes of ascidians which are expressed in the protein- secreting zones and encode novel secretory proteins presumably used for trapping food (Ogasawara et al. 1996; Ogasawara and Satoh 1998). In addition, we have cloned ascidian homologs of *TTF-1* and *TPO* which are expressed in non-overlapping patterns in the endostyle (Ogasawara et al. 1999b). Therefore, it does not appear that the ascidian TTF-1 direct regulates the expression of *TPO* in the adult endostyle as it does in vertebrates. In the present study, to determine whether TTF-1 regulates *TPO* gene in the amphioxus endostyle, I isolated and characterized the homologs of amphioxus *TTF-1* and *TPO* from *Branchiostoma belcheri*. Both genes are expressed in the adult endostyle, in partially overlapping patterns. Therefore, the present study provides the first evidence of overlapping expression of *TTF-1* and *TPO* in the endostyle of lower chordates.

Materials and Methods

Biological materials

Specimens of *Branchiostoma belcheri* were collected during the spawning season at Ariake-Kai near the Aizu Marine Biological Station of Kumamoto University, Kyushu, Japan, and at Gokasyo-bay near the National Research Institute of Aquaculture, Mie, Japan. Naturally spawned eggs were fertilized and raised at room temperature. They developed into gastrulae, neurulae and larvae, about 5, 10 and 48 h after fertilization, respectively. Samples at appropriate developmental stages were fixed for in situ hybridization. Adult specimens were sectioned transversely at about 3- mm intervals and immediately fixed for in situ hybridization. These specimens were kept at -20°C until use.

Adult specimens and dissected endostyles were frozen in liquid nitrogen for RNA isolation and kept at -80°C until use.

Isolation and sequencing of cDNA clones for amphioxus TTF-1 and TPO genes

The sense-strand oligonucleotide primer for TTF-1, NKX-F1: 5'-TT(CT)AG(CT)CA (AG)GCNCA(AG)GTNTA(CT)GA(AG)(CT)T-3', which corresponds to the amino acid sequence FSQAQVYEL, and the antisense oligonucleotide primer NKX-R: 5'-(GT)TT (CT)TG(AG)AACCA(AGT)AT(CT)TTNAC(CT)TG-3', which corresponds to the amino acid sequence QVKIWFQN, were synthesized based on conserved NK-2 type homeodomains from *C. elegans, Drosophila,* tunicates, amphioxus and vertebrates. Target fragments were amplified by RT-PCR from adult endostyle total RNA and 131- bp PCR product was obtained. Oligonucleotide primers for TPO, TPO-F: ACIGCIGCITT(TC)(CA)GITT (TC)GGICA, which corresponds to the amino acid sequence TAAFRFGH, and the antisense oligonucleotide TPO-R: GGIA(AG)ICC(AG)TG(AG)TCIC(GT)ICCIC(GT)(TC)TG, which corresponds to the amino acid sequence QRGRDHGLP, were synthesized based on conserved region of the TPO. Target fragments were amplified by RT-PCR from adult endostyle total RNA and 308-bp PCR product was obtained. These PCR fragments were randomly labeled with [^{32}P]dCTP (Amersham), and 3.0 x 10^5 phages of a mixed cDNA

library of *B. belcheri* adult and gastrula (Terazawa and Satoh 1997) were screened under hybridization condition of 6x SSPE, 0.1% SDS, 1x Denhardt's solution, 50% formamide at 42°C for 16 h, and washing condition of 2x SSC-0.1% SDS at 55°C for 30 min., 1x SSC-0.1% SDS at 55°C for 30 min, 0.1x SSC-0.1% SDS at 55°C for 30 min. Isolated clones were sequenced using an ABI PRISM 377 DNA Sequencer (Perkin Elmer).

Sequence Comparisons and Molecular Phylogenetic Analyses

Sequences were aligned using the SeqQpp 1.9 manual aligner for Macintosh (Gilbert 1993). Phylogenetic analyses were performed on the amino acid sequences of the homeodomain. Estimation of molecular phylogeny was carried out by the neighbor-joining method (Saitou and Nei 1987) using the CLUSTAL V (Higgins et al. 1992) program. Confidence in the phylogeny was assessed by bootstrap resampling of the data (x 1,000) (Felsenstein 1985).

Northern blot analysis

Total RNA was isolated by the AGPC method (Chomczynski and Sacchi 1987), and poly(A)$^+$ RNA was purified with Oligotex dT30 beads. Northern blot hybridization was carried out using standard procedures (Sambrook et al. 1989) and filters were washed under high-stringency conditions (hybridization: 6x SSPE, 0.1% SDS, 1x Denhardt's solution, 50% formamide at 42°C for 16 h; washing: 2x SSC-0.1% SDS at 60°C for 30 min., 1x SSC-0.1% SDS at 60°C for 30 min, 0.1x SSC-0.1% SDS at 60°C for 30 min). Entire region of cDNAs were labeled with [^{32}P]-dCTP using a random primed labeling kit (Boehringer Mannheim) for hybridization probes.

RT-PCR/Southern blot analysis

Total RNA of various tissues (endostyle, gill, intestine, body-wall muscle, notochord, and nerve cord) was extracted from small pieces of adult tissues that were sonicated in an

extraction buffer containing guanidinium-thiocyanate. Total RNA was isolated from the extracts by protease K and phenol-chloroform treatment. Ten μg of total RNA was reverse-transcribed after hybridization with oligo dT primer, and then PCR (30 cycles: 1 min at 94°C, 2 min at 50°C and 1 min at 72°C) was carried out with specific primers. Primers for *BbTTF-1* are BbTTF-1F; 5'- CTAGTCAAAGACGGCAAGCCG-3' and BbTTF-1R; 5'-TGCTACAA TACTGGCACGTCC-3', and for *BbTPO* are BbTPOF; 5'- GAGCAGTTCAAGGCATATCG C-3' and BbTPOR; 5'-CATAATGACGCTGTACCGTGC-3'. Amplified fragments were blotted onto Hybond-N+ nylon membranes (Amersham). The blots were hybridized and washed under the same conditions as for Northern blotting.

In situ hybridization

Whole-mount in situ hybridization was carried out essentially as described by Holland et al. (1992). Embryos and transversely sectioned specimens of adults were fixed in 4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS buffer at 4°C for 12 h. Probes were synthesized by following the instructions from the supplier of the labeling kit (DIG RNA Labeling kit; Boehringer Mannheim). Some specimens were embedded in polyester wax (BDH Chem. Ltd.) and sectioned at 10 μm intervals for observation at high magnification.

Results

Isolation and characterization of cDNA clones for *TTF-1(Nkx-2.1)* gene of the amphioxus *Branchiostoma belcheri*

The longest cDNA clone included 2,353 nucleotides excluding the poly (A) tail, and as is evident from the Northern blotting shown in Fig. 4A, this cDNA covered the full-length sequence of the gene transcript. This sequence contained a single open reading frame that encoded a polypeptide of 359 amino acids, and the molecular mass (Mr) of the predicted protein was 39.5 kDa. This cDNA clone encoded a conserved TN domain, NK-2- type homeodomain and NK2 domain (Figs. 1, 2), and therefore we named this gene *BbTTF-1* (*Branchiostoma belcheri TTF-1*).

Figure 1 compares the amino acid sequence of BbTTF-1 to that of TTF-1 (AmphiNk2-1) from another species of *Branchiostoma*, *B. floridae* (Venkatesh et al. 1999). The amino acid sequences of BbTTF-1 and AmphiNk2- 1 was very similar (98.3%), and TN domains (underlined), NK-2- type homeodomain (boxed) and NK2 domain (dotted underlined) are identical. Furthermore, the nucleotide sequences of ORFs and 5' UTRs were highly conserved (93.9% and 98.9% identical, data not shown) between the genes, and the 3' UTR sequences also closely resemble each other (73.8% identical). The overall identity of the entire DNA sequence was 86.4%.

Sequence comparison and molecular relationships of TTF-1 proteins

Figure 2 shows that the TN domains, homeodomains and NK-2 domains of the TTF-1 are highly conserved among the chordates (over 90% identity). On the other hand, BbTTF-1 showed a lower level of amino acid sequence identity compared with other NK-2- type proteins (Nkx-2.2, Nkx-2.3 and Nkx-2.5).

The evolutionary relationships of the TTF-1 proteins were examined by molecular phylogenetic analyses (Fig. 3). A molecular phylogenetic tree was constructed by the neighbor-joining method based on the amino acid sequence of the homeobox domain. *Drosophila* NK1 was used as an outgroup. As is evident from this tree, amphioxus BbTTF-1

forms a group with Nkx-2.1 (mouse TTF-1/Nkx-2.1, human TTF-1/Nkx-2.1, dog Nkx-2.1, chick Nkx-2.1, amphioxus AmphiNK2-1, and ascidian CiTTF-1 and HrTTF-1). In addition, this group is included in a larger clade with *Drosophila* NK2, *C. elegans* CEH-22 (Okkema and Fire 1994), mouse Nkx-2.2 (Price et al. 1992) and amphioxus Nkx-2.2 (Holland et al. 1999). This larger clade was supported by a bootstrap value of 79%. From these data, together with the results mentioned above, we conclude that BbTTF-1 is a member of the Nkx-2.1 family.

BbTTF-1 is expressed in the endostyle

The expression of *BbTTF-1* was examined by Northern blot analysis, RT-PCR/Southern blot analysis and in situ hybridization. Northern blots using poly(A)$^+$ RNA prepared from the ventral and dorsal halves of adults detect a single band of *BbTTF-1* transcript of about 2.4 kb only in the ventral half of the adult (Fig. 4A). The length of the transcript roughly coincided with that of the longest cDNA characterized. Spatial expression of *BbTTF-1* in the adult tissues was also examined by RT-PCR/Southern blot analysis. The amplified product of the *BbTTF-1* transcript was detected only in the endostyle, and there was no signal in gill, intestine, muscle, notochord, and nerve cord (Fig. 4B). On the other hand, transcripts of the *B. belcheri* cytoplasmic actin gene were detected in all tissues, including notochord, nerve cord, body-wall muscle, intestine, pharyngeal gill and endostyle (data not shown). Therefore, transcripts of *BbTTF-1* were restricted to the endostyle of adult amphioxus.

In situ hybridization shows that expression of *BbTTF-1* in embryos and early larvae is like that previously described for another amphioxus *TTF-1* gene, *AmphiNk2-1*. *BbTTF-1* expression at the neurula stage (Fig. 4C) is in the anterior endoderm (arrowhead) and the anterior part of the neural tube (twin arrowheads). In the late neurula (Fig. 4D), *BbTTF-1* was mainly expressed on the right side of the pharynx in the developing endostyle (arrowhead) and weak expression was observed in the middle and posterior parts of the gut (arrows). At the larval stage (Fig. 4E), *BbTTF-1* was mainly expressed in the endostyle primordium (arrowhead). Very weak expression was also observed in the anterior part of the neural tube (twin arrowheads) and in the hindgut (arrow).

Adult specimens sectioned at about 3- mm intervals across the longitudinal axis were also examined. For the sense probe as control (Fig. 4F), only a weak background signal was

detected. The *BbTTF-1* antisense probe showed expression only in the endostyle (Fig. 4G, red arrowhead). No other signals except for background signals were detected. In order to examine the *BbTTF-1* expression pattern in the endostyle in detail, the endostyle was sectioned transversely and observed at high magnification. As shown in Fig. 4H, *BbTTF-1* expression was detected in all regions of the endostyle (zones 1 to 6), with particularly strong signals being detected in zones 5 and 6. In some specimens, strong *BbTTF-1* expression was also detected additionally in zone 1 (arrowhead).

Isolation and characterization of cDNA clone for amphioxus *TPO* gene

Eight cDNA clones for *BbTPO* (*Branchiostoma belcheri* thyroid peroxidase) were isolated. The longest contained 3,449 nucleotides excluding the poly (A) tail with an open reading frame that encoded a polypeptide of 741 amino acids.

cDNA clones of TPO genes have been isolated from several vertebrates and ascidians as well as amphioxus: human (Kimura et al. 1989), mouse (Kotani et al. 1993), and the ascidians *Ciona intestinalis* and *Halocynthia roretzi* (Ogasawa et al. 1999b). Overall, the amino acid identities of BbTPO and TPO proteins of other chordates are between 41.7% and 46.9%. On the other hand, BbTPO shows less identity when compared with non-thyroid-type peroxidase: its identity with human salivary peroxidase is 38.3% (Kiser et al. 1996), with human eosinophil peroxidase 38.8% (Ten et al. 1989), with human myeloperoxidase 40.7% (Hashinaka et al. 1988), and with sea urchin ovoperoxidase 32.9% (LaFleur et al. 1998) (data not shown).

While all of the amino acids required for peroxidase activity (asterisks in Fig. 5) are completely conserved, the BbTPO lacks the hydrophobic region of the carboxy-terminus like the case of the non-thyroid- type peroxidases, including salivary peroxidase, eosinophil peroxidase, myeloperoxidase and ovoperoxidase (data not shown).

Expression of the amphioxus *BbTPO* gene in the endostyle

Northern blot analysis revealed the presence of a single *BbTPO* transcript of 3.5 kb (Fig. 6A)

in the ventral half, but not the dorsal half of adults. RT-PCR/Southern blot analysis of adult tissues showed *BbTPO* transcripts in the endostyle and pharyngeal gills only (Fig. 6B).

Whole- mount in situ hybridization did not detect *BbTPO* expression in embryos and larvae (data not shown). In adults, *BbTPO* expression was limited to the endostyle (Fig. 6C, red arrowheads). In some specimens, *BbTPO* expression was also detected in the outer wall of the pharyngeal gills (Fig. 6D, yellow arrowheads). To further clarify the latter expression pattern of *BbTPO*, adult amphioxus were partially dissected from the dorsal side and prepared as whole- mounts for in situ hybridization. Figure 6E show two stripes of *BbTPO* expression in the endostyle (red arrowheads) extending from the forepart of the pharynx to the intestine. *BbTPO* expression in the pharyngeal gills was also detected in the posterior part of the pharynx near the intestine (yellow arrowheads). Sections show the *BbTPO* expression mainly in zones 5 and 6 (Fig. 6F, red arrowheads). In some specimens, *BbTPO* expression was detected in the outer wall of the gill bars (yellow arrowheads), and in zones 1 and 3.

Discussion

TTF-1 and *TPO* are key molecules in evolution of the thyroid

The endostyle, which concentrates iodine and has peroxidase activity, is generally considered to be homologous to the thyroid gland follicle of higher vertebrates (Barrington 1957; Salvatore 1969; Thorpe et al. 1972; Dunn 1974, 1980; Fujita and Sawano 1979). Several mammalian thyroid follicle-specific molecules have been characterized, and thyroid-specific gene expression as well as gene regulation mechanisms have been analyzed in relation to thyroid development and function (Civitareale et al. 1989; Lazzaro et al. 1991; Plachov et al. 1990; Zannini et al. 1997).

One of these molecules, *TTF-1* encodes a transcription factor with an NK-2- type homeodomain for DNA binding, and is member of the *Nkx-2.1* gene family (Harvey 1996). In the mammalian thyroid follicle, TTF-1 binds directly to the sequences upstream of the *TPO, Tg* and thyrotropin receptor genes, and regulates their expression (Damante et al. 1994; Civitareale et al. 1993; Mascia et al. 1997). Homologs of vertebrates *Nkx-2.1* have been isolated from two ascidian species, *C. intestinalis* (Ristoratore et al. 1999) and *H. roretzi* (Ogasawara et al. 1999a) and from amphioxus *B. floridae* (*AmphiNK2-1*; Venkatesh et al. 1999). In the present study, a homolog (*BbTTF-1*) was isolated from another amphioxus *B. belcheri*. *BbTTF-1* encodes a typical NK-2- type homeodomain, and phylogenetic analysis indicated that *BbTTF-1* is a member of the *Nkx-2.1* gene family. The amino acid sequence of BbTTF-1 is highly conserved (98.3% identical) compared with that of AmphiNk2-1. The nucleotide sequences of the two genes also closely resemble each other.

The embryonic and larval expression pattern of *BbTTF-1* is identical to that of *AmphiNk2-1*. During embryogenesis, both genes are expressed in embryonic gut, nerve cord and endostyle (Venkatesh et al. 1999). RT-PCR/Southern blotting and in situ hybridization revealed that *BbTTF-1* expression in adults is restricted to the endostyle. These results suggest that amphioxus *TTF-1* is associated with endostyle development and function. Thus, *TTF-1* is appears to be a key molecule for understanding the development of the thyroid gland follicle and the endostyle.

On the other hand, *TPO* encodes an enzyme involved in iodinating Tg (thyroglobulin) and is therefore important for thyroid hormone synthesis. This gene is considered to be a specific

marker for differentiation of the thyroid gland follicle (Francis-Lang et al. 1992). cDNA clones of *TPO* have been isolated from several mammals (humans, mice, rats and pigs) and their structures and functions have been well characterized. *TPO* homologs have been isolated from ascidians (Ogasawara et al. 1999b) and amphioxus (*BbTPO*, in the present study). The amino acids which are associated with peroxidase activity (Taurog and Wall 1998) are completely conserved among the various known TPOs. Thus, not only ascidian TPOs but also amphioxus TPO might have peroxidase activity. Interestingly, the amphioxus TPO has no long hydrophobic carboxy-terminal tail which is thought to be required for membrane binding. Other peroxidases, such as lactoperoxidase (Dull et al. 1990) and myeloperoxidase, also lack this hydrophobic tail. Therefore, during the evolution from common ancestor of ascidian and amphioxus to the amphioxus *B. belcheri*, this characteristic tail might have been lost. However, whether TPO of other amphioxus species have a hydrophobic tail or not should be examined in other amphioxus species.

The strong expression of *BbTPO* in the adult endostyle was restricted to zone 5, and weak expression is detected in zone 6. This expression pattern coincides with that of histochemical peroxidase activities reported by Tsuneki et al. (1983), Fredriksson et al. (1984) and Ericson et al. (1985). In some individuals, very weak expression was also detected in zones 1 and 3, and strong expression in the outer wall of the pharyngeal gill. These conserved sequence and expression pattern of *BbTPO* in the adult endostyle supports the idea that the endostyle is homologous to the vertebrate thyroid gland follicle. Therefore, *TPO* should be a useful molecule for analyzing the origin and evolution of thyroid function in the endostyle.

In addition, the overlapping expression of *BbTTF-1* and *BbTPO* suggests the possibility that TTF-1 regulates the *TPO* expression in amphioxus. The amino acid sequences, gene expression patterns and gene expression mechanisms of *TTF-1* and *TPO* are comparable between vertebrates and lower chordates. Therefore, these molecules will be useful not only for analyzing their functions in the endostyle but also for analyzing the molecular mechanisms involved in evolution of the thyroid-related function.

Evolution of the mechanism of regulation of thyroid-related gene expression

Previous studies of ascidian *TTF-1*s (*CiTTF-1* and *HrTTF-1*) and *TPO*s (*CiTPO* and *HrTPO*) indicated that these genes are expressed specifically in the endostyle. Observation of

specimens at high magnification by in situ hybridization revealed that ascidian *TTF-1*s are mainly expressed in zones 1, 3 and 5 of the supporting elements. On the other hand, the ascidian *TPO*s were expressed in zone 7, which is one of the thyroid-equivalent elements. Therefore, the expression patterns of the *TTF-1* and *TPO* genes do not overlap in the ascidian endostyle, suggesting that ascidian TTF-1 does not directly regulate ascidian *TPO* expression in the endostyle.

The organization of the amphioxus endostyle resembles that of the ascidian endostyle. The amphioxus endostyle is divided into six zones. From studies of the histochemical activities of peroxidase (Tsuneki et al. 1983; Fredriksson et al. 1984) and iodine-concentrating activity (Thorpe et al. 1972; Dunn 1974; Fredriksson et al. 1984; Ericson et al. 1985), the thyroid-equivalent regions are thought to be zones 5 and 6. The present study demonstrated that both amphioxus *TTF-1* and *TPO* are expressed in the endostyle, and the expression domains overlap in zones 5 and 6. This result provides the first molecular evidence for overlapping expression of *TTF-1* and *TPO* in lower chordates, suggesting that this overlapping expression first appeared during the evolution to cephalochordates. However, some of the *BbTPO* expression domains do not coincide with the *BbTTF-1* expression domains. Especially, the *BbTPO* expression domain in the outer wall of the pharyngeal gills does not overlap with the *BbTTF-1* expression domain. Therefore, in the pharyngeal gills, *BbTPO* expression might not be regulated by BbTTF-1. Furthermore, the occurrence of a *BbTTF-1* expression region in which there is no *BbTPO* expression suggests that BbTTF-1 is not an obligatory activator for *BbTPO*. Other molecules may be able to activate *BbTPO* gene expression. An alternative idea is that the expression domains of *BbTTF-1* and *BbTPO* are merely overlapping, and BbTTF-1 is not associated with regulation of the *BbTPO* expression. In order to analyze the regulation of *BbTPO* expression, the upstream *cis*-regulatory sequences of *BbTPO* and possible gene-regulation-related interactions between TTF-1 and *TPO* should be examined. In any case, because *BbTTF-1* and *BbTPO* are expressed in the same zone of the amphioxus endostyle, possible gene-regulation-related interaction between TTF-1 and TPO should be examined.

Studies of the mammalian thyroid gland follicle have shown that several structural proteins and transcription factors are associated with the development and function of the thyroid gland. In mice, transcription factors TTF-1, TTF-2 and Pax8 bind to the upstream regulatory sequences of the *Tg*, *TPO* and *thyrotropin receptor* genes to regulate their expression (Civitareale et al. 1989, Francis-Lang et al. 1992; Zannini et al. 1992; Zannini et al. 1997).

Recently, amphioxus *B. floridae* homologs of *TTF-1* (*AmphiNk2-1*; Venkatesh et al. 1999) and *Pax8* (*AmphiPax2/5/8*; Kozmik et al. 1999) were isolated and characterized. The expressin of these genes was detected in the developing and larval endostyle. Furthermore, an ascidian homolog of *Pax8* (*HrPax2/5/8*; Wada et al. 1998) was also expressed in the adult endostyle (personal communication). Therefore, these amphioxus and ascidian homologs might provide further knowledge for understanding the origin and evolution of the mechanism of gene regulation of thyroid-related molecules.

The lamprey is one of the most primitive vertebrates, and its larva also has a typical endostyle. This organ consists of several cell types, i.e., 1v, 1d, 2a, 2b, 2c, 3, 4 and 5 (Egeberg 1965; Fujita and Honma 1968). Histologically, the organization differs from that of the endostyle of ascidians and amphioxus. However, iodine-concentrating activity was reported in cells of types 2c and 3 (Fujita and Honma 1969), and peroxidase activity in cells of types 2c and 3 (Tsuneki et al. 1983). These cells are located dorso-laterally to the glandular region as in the case of other endostyles. Therefore, the types 2c and 3 cells are thought to be the thyroid-equivalent cells. Furthermore, transformation of the endostyle to the thyroid gland follicle during metamorphosis was confirmed histologically (Wright and Youson 1976). It will be very interesting to examine expression and mechanisms of gene regulation of *TTF-1* and *TPO* in the endostyle and thyroid gland follicle during lamprey metamorphosis.

Origin and evolution of the thyroid-related activities in the endostyle

To date, thyroid-related activities including iodine-concentrating activity and thyroid peroxidase activity, have been reported in various organisms: ascidians (sessile tunicates; Ascidiacea), amphioxus, larval lampreys, and also pelagic tunicates (Fredriksson et al. 1985) including *Oikopleura dioica* (Appendicularia), *Salpa fusiformis* (Desmomyaria) and *Dolioletta gegenbauri* (Cyclomyaria). The endostyle of all of these organisms contains large glandular regions and thyroid-related regions. However, the structures of the endostyles in these organisms vary depending on the organism. The number of cell types and shapes of the cells are not the same. Thyroid-related cells contain both iodine- concentrating activity and peroxidase activity, and are uniformly located in a similar position (dorso-laterally to the glandular region). Therefore, the position of the thyroid-related cells might have been established during the evolution of the urochordates. On the other hand, the thyroid-related functions of iodine concentration and iodoamino acid formation have been reported not only in

chordates, but also in echinoderms and hemichordates (reviewed by Eales 1997). Therefore, iodine metabolism appeared at least by the time of evolution to chordates.

Acorn worms (hemichordates) are key organisms for analyzing the origin and evolution of the chordate body plan, because acorn worms are the most primitive deuterostomes, and they have remarkable gill slits. Acorn worms have no endostyle, but iodine- concentrating activity in the surface of the glandular cells and iodoamino acid has been reported (Gorbman et al. 1954). Molecular phylogenetic analysis using *TTF-1* and *TPO* should be performed in hemichordates, and should provide helpful knowledge of the origin and evolution of the thyroid-related molecules and thyroid formation.

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Figure legends

Fig. 1 Predicted amino acid sequences of thyroid transcription factor-1 gene product (BbTTF-1) of *B. belcheri* (top), and sequence comparison with AmphiNk2-1 of *B. floridae* (under, Venkatesh et al. 1999). The polypeptide encoded by BbTTF-1 contains 359 amino acids, and the molecular mass is estimated to be 39.5 kDa. The nucleotide sequence of *BbTTF-1* will appear under the DDBJ/EMBL/GenBank accession number AB028842. Amino acids identical between BbTTF-1 and AmphiNk2- 1 are indicated with asterisks (98.3%). The TN domain (underlined), NK-2- type homeodomain (boxed) and NK-2 domain (dotted underlined) are identical in BbTTF-1 and AmphiNk2- 1.

Fig. 2A, B Comparison of the amino acid sequences of the TN domain (A) and homeodomain (B). Human TTF-1 (Saiardi et al. 1995), mouse TTF-1 (Oguchi et al. 1995), amphiNk2- 1 (Venkatesh et al. 1999), HrTTF-1 (Ogasawara et al. 1999a), CiTTF-1 (Ristoratore et al. 1999), amphiNk2- 2 (Holland et al. 1999), mouse Nkx-2.2 (Price et al. 1992), mouse Nkx-2.3 (Price et al. 1992), mouse Nkx-2.5 (Lints et al. 1993), and *Drosophila* NK1-NK4 (Kim and Nirenberg 1989). The percentage of identity is shown on the right side. The dots represent identical amino acids. All NK-2- type homeodomains (TTF-1/Nkx-2.1, Nkx-2.2, Nkx-2.3, Nkx-2.4, Nkx-2.5, NK2, NK3 and NK4) share a tyrosine residue at position 54 within the domain (arrow).

Fig. 3 Phylogenetic relationships among TTF-1 (Nkx-2.1) proteins. The tree was constructed by means of the neighbor-joining method using the amino acid sequences of the homeodomain. The numbers at the branches are bootstrap percentages (only those over 50% are shown).

Fig. 4A-H. Distribution of *BbTTF-1* transcript in *B. belcheri*. **A** Northern blots of 5 μg of poly(A)$^+$ RNA prepared from the dorsal half (dh) and ventral half (vh) of adults. **B** RT-PCR/Southern blot analysis of *BbTTF-1* expression. Notochord (Not), nerve chord (NC), body-wall muscle (BWM), intestine (Int), pharyngeal gill (PhG), and endostyle (En). **C-E** Localization of *BbTTF-1* transcript in embryos and larvae as revealed by in situ hybridization. Scale bars, 100 μm. **C** At the neurula stage, the transcript is detected in the endodermal

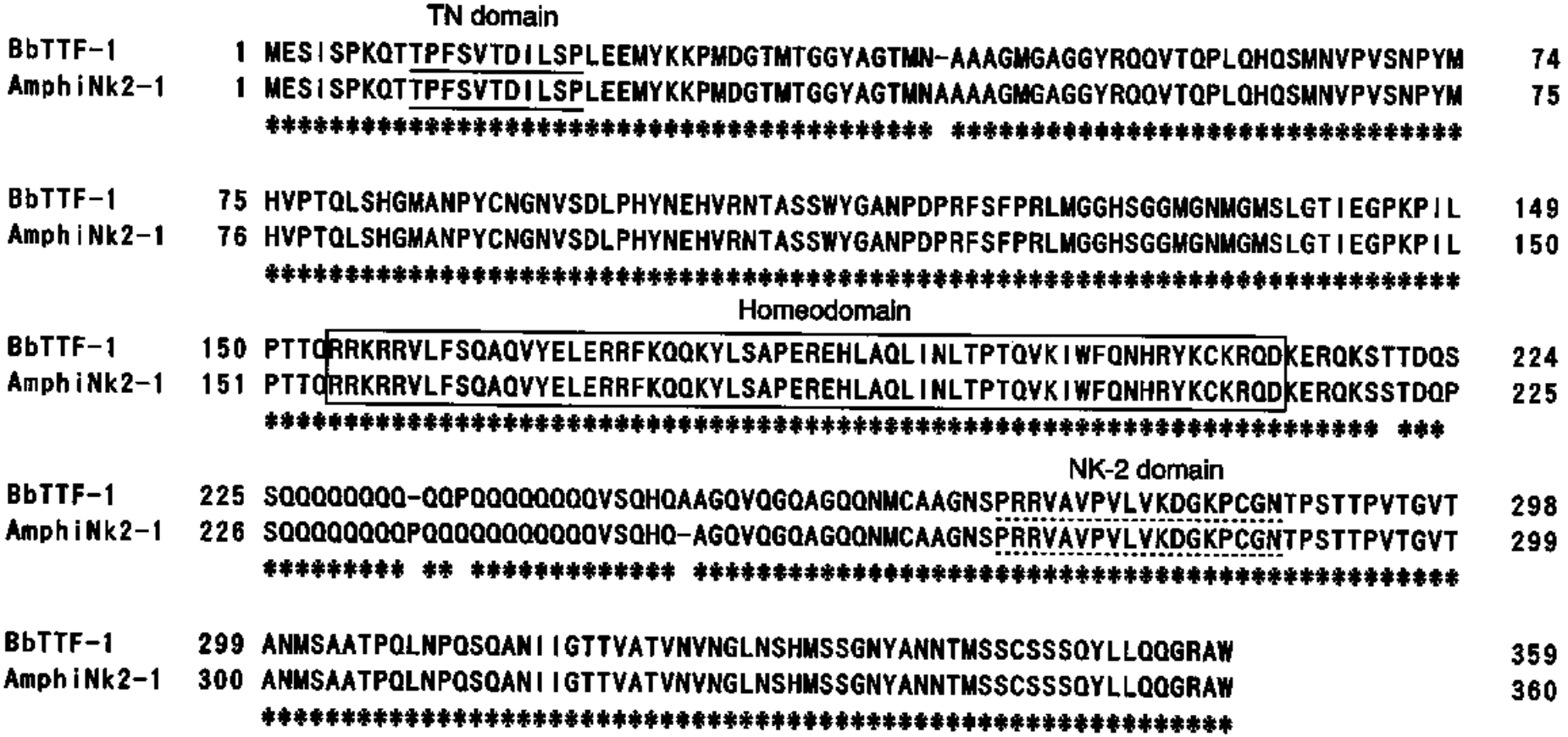
region (arrowhead) and anterior part of the neural tube (twin arrowheads). **D** At the late neurula stage, the transcript is detected in the pharynx (arrowhead), and middle and posterior part of the gut (arrows). **E** In larva, transcript is detected in the endostyle primordium (arrowhead) and hindgut (arrow). Weak expression in the anterior part of the neural tube (twin arrowheads) is also detected. **F-H** Expression of *BbTTF-1* in the adult amphioxus. Sectioned adult specimens are hybridized as whole mount specimen with *BbTTF-1* sense probe as a negative control (**F**) and with antisense probe (**G**). No signals above background are detected in **F**. A distinct hybridization signal is detected only in the endostyle (red arrowhead in **G**). Scale bars in **F** and **G**, 1 mm. **H** The endostyle is sectioned transversely and observed at high magnification. Scale bars, 100 μm. Signals for *BbTTF-1* expression are detected throughout the endostyle, with very strong signals in zones 5 and 6. In some specimens, strong signals for *BbTTF-1* expression are also detected in zone 1 (arrowhead). En, endostyle; PhG, pharyngeal gill; Ph, pharynx; Not, notochord; BWM, body-wall muscle; and NC, nerve cord.

Fig. 5 Comparison of the amino-acid sequences of TPO proteins. BbTPO encoded a protein of 741 amino acids and calculated molecular mass (Mr) was 82.7 kDa. Nucleotide and predicted amino acid sequences of *BbTPO* will appear under the DDBJ/EMBL/GenBank accession number AB028841. Amino acids identical with those of CiTPO (Ogasawara et al. 1999b), HrTPO (Ogasawara et al. 1999b), human TPO (Kimura et al. 1989), and mouse TPO (Kotani et al. 1993) are boxed. The overall identity between the amino acid sequences of BbTPO and the TPO- type peroxidases are: human TPO, about 43.4%, mouse, TPO, about 41.7%, CiTPO, about 46.9%, and HrTPO, about 46.1%; these identities are higher than those between BbTPO and non-TPO type peroxidase [human salivary peroxidase (38.3%), human eosinophil peroxidase (38.8%), human myeloperoxidase (40.7%), and sea urchin ovoperoxidase (33.8%)]. BbTPO lacks a carboxy-terminal tail. All amino acids required for peroxidase activity are completely conserved (asterisks).

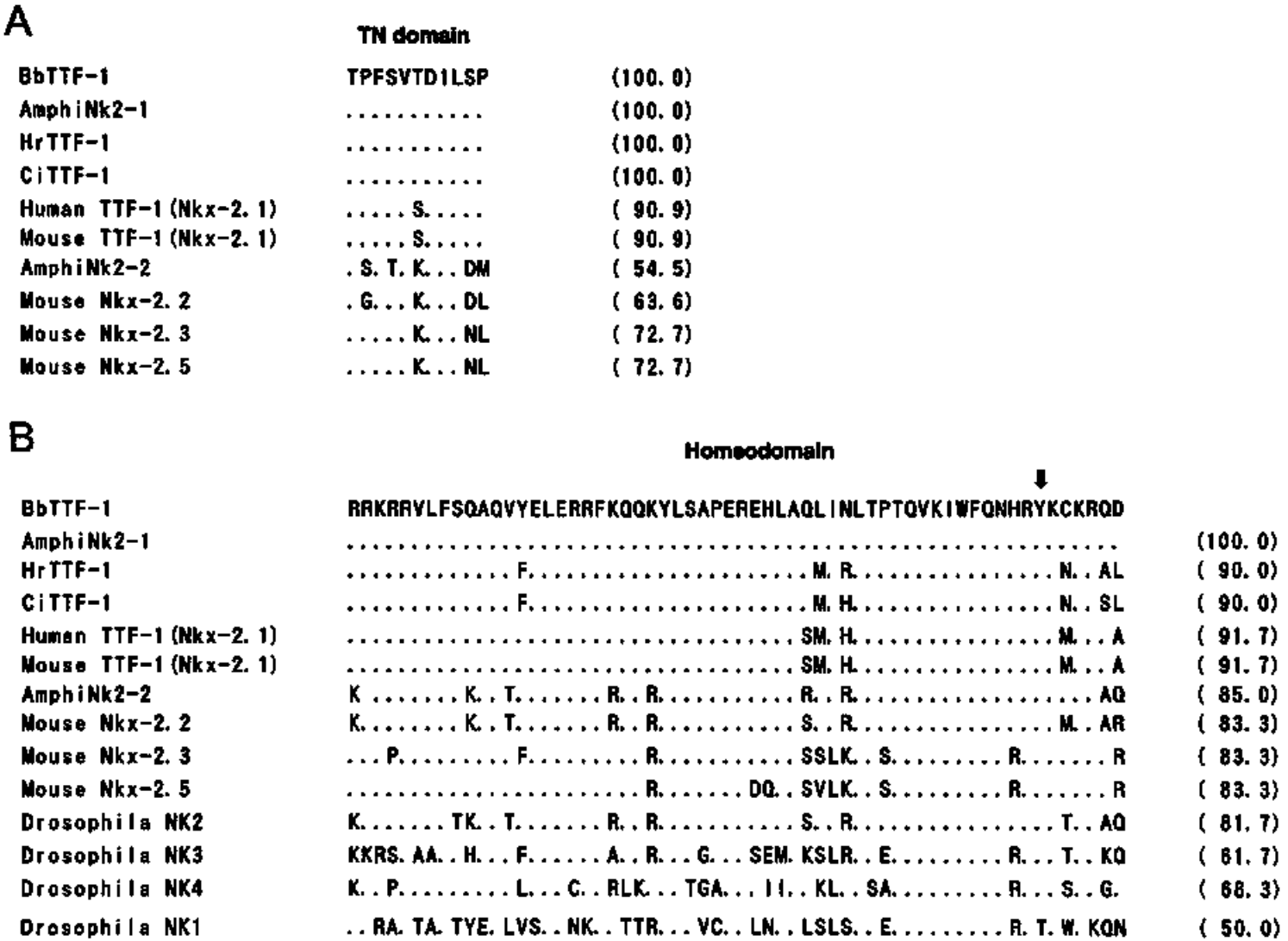
Fig. 7A-F Distribution of *BbTPO* transcript in *B. belcheri*. **A** Northern blots of poly(A)$^+$ RNA prepared from the dorsal half (dh) and ventral half (vh) of adults. **B** RT-PCR /Southern blot analysis of *BbTPO* expression. **C-F** Expression of *BbTPO* in the adult amphioxus revealed by in situ hybridization. **C, D** Sectioned adult whole mount specimens are hybridized with *BbTPO* antisense probe. Scale bars, 1 mm. Two stripes of

hybridization signals are detected in the endostyle (red arrowheads). In some specimens hybridization signals were also detected in the outer wall of the pharyngeal gill bars (D, yellow arrowheads). E Adult specimens are dissected partially from the dorsal side and viewed from a dorsal position. Two stripes of *BbTPO* signal are detected in the endostyle (red arrowheads), and also in the posterior part of the pharyngeal gill (yellow arrowheads). Scale bars, 1 mm. F The endostyle is sectioned transversely and observed at high magnification. Scale bar, 100 μm. The *BbTPO* signal is detected in zones 5 and 6 (red arrowheads), and also detected in the outer wall of the pharyngeal gill (yellow arrowheads). A very weak signal is detected in zones 1 and 3. En, endostyle; PhG, pharyngeal gill; Int, intestine; and Not, notochord.

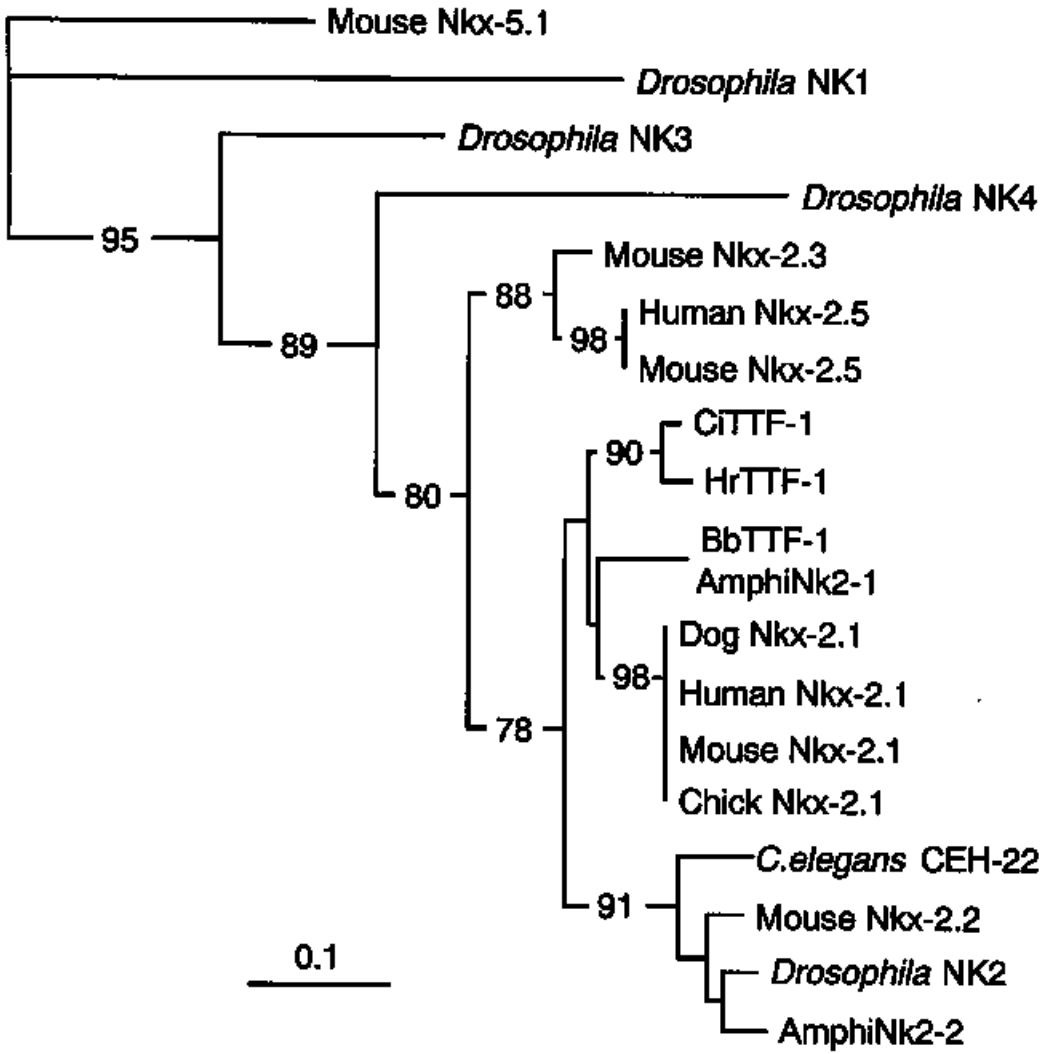
ure 1


TN domain
BbTTF-1 1 MESISPKQTTPFSVTDILSPLEEMYKKPMDGTMTGGYAGTMN-AAAGMGAGGYRQQVTQPLQHQSMNVPVSNPYM 74
AmphiNk2-1 1 MESISPKQTTPFSVTDILSPLEEMYKKPMDGTMTGGYAGTMNAAAAGMGAGGYRQQVTQPLQHQSMNVPVSNPYM 75
BbTTF-1 75 HVPTQLSHGMANPYCNGNVSDLPHYNEHVRNTASSWYGANPDPRFSFPRLMGGHSGGMGNMGMSLGTIEGPKPIL 149
AmphiNk2-1 76 HVPTQLSHGMANPYCNGNVSDLPHYNEHVRNTASSWYGANPDPRFSFPRLMGGHSGGMGNMGMSLGTIEGPKPIL 150
Homeodomain
BbTTF-1 150 PTTQRRKRRVLFSQAQVYELERRFKQQKYLSAPEREHLAQLINLTPTQVKIWFQNHRYKCKRQDKERQKSTTDQS 224
AmphiNk2-1 151 PTTQRRKRRVLFSQAQVYELERRFKQQKYLSAPEREHLAQLINLTPTQVKIWFQNHRYKCKRQDKERQKSSTDQP 225
NK-2 domain
BbTTF-1 225 SQQQQQQQQ-QQPQQQQQQQQVSQHQAAGQVQGQAGQQNMCAAGNSPRRVAVPVLVKDGKPCGNTPSTTPVTGVT 298
AmphiNk2-1 226 SQQQQQQQQPQQQQQQQQQQQVSQHQ-AGQVQGQAGQQNMCAAGNSPRRVAVPVLVKDGKPCGNTPSTTPVTGVT 299
BbTTF-1 299 ANMSAATPQLNPQSQANIIGTTVATVNVNGLNSHMSSGNYANNTMSSCSSSQYLLQQGRAW 359
AmphiNk2-1 300 ANMSAATPQLNPQSQANIIGTTVATVNVNGLNSHMSSGNYANNTMSSCSSSQYLLQQGRAW 360

gure 2


A
TN domain
BbTTF-1 TPFSVTDILSP (100.0)
AmphiNk2-1 ..... (100.0)
HrTTF-1 ..... (100.0)
CiTTF-1 ..... (100.0)
Human TTF-1 (Nkx-2.1) .....S..... (90.9)
Mouse TTF-1 (Nkx-2.1) .....S..... (90.9)
AmphiNk2-2 .S.T.K..DM (54.5)
Mouse Nkx-2.2 .G..K..DL (63.6)
Mouse Nkx-2.3 .....K..NL (72.7)
Mouse Nkx-2.5 .....K..NL (72.7)
B
Homeodomain
BbTTF-1 RRKRRVLFSQAQVYELERRFKQQKYLSAPEREHLAQLINLTPTQVKIWFQNHRYKCKRQD
AmphiNk2-1 ..... (100.0)
HrTTF-1 .....F.....M.R.....N..AL (90.0)
CiTTF-1 .....F.....M.H.....N..SL (90.0)
Human TTF-1 (Nkx-2.1) .....SM.H.....M..A (91.7)
Mouse TTF-1 (Nkx-2.1) .....SM.H.....M..A (91.7)
AmphiNk2-2 K.....K..T.....R..R.....R..R.....AQ (85.0)
Mouse Nkx-2.2 K.....K..T.....R..R.....S..R.....M..AR (83.3)
Mouse Nkx-2.3 ...P.....F.....R.....SSLK..S.....R.....R (83.3)
Mouse Nkx-2.5 .....R.....DQ..SVLK..S.....R.....R (83.3)
Drosophila NK2 K.....TK..T.....R..R.....S..R.....T..AQ (81.7)
Drosophila NK3 KKRS.AA..H...F.....A..R...G...SEM.KSLR..E.....R...T..KQ (61.7)
Drosophila NK4 K..P.....L...C..RLK...TGA....I..KL..SA.....R...S..G. (66.3)
Drosophila NK1 ..RA.TA.TYE.LVS..NK..TTR...VC..LN..LSLS..E.....R.T.W.KQN (50.0)

gure 3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Drosophila NK1
Drosophila NK3
Drosophila NK4
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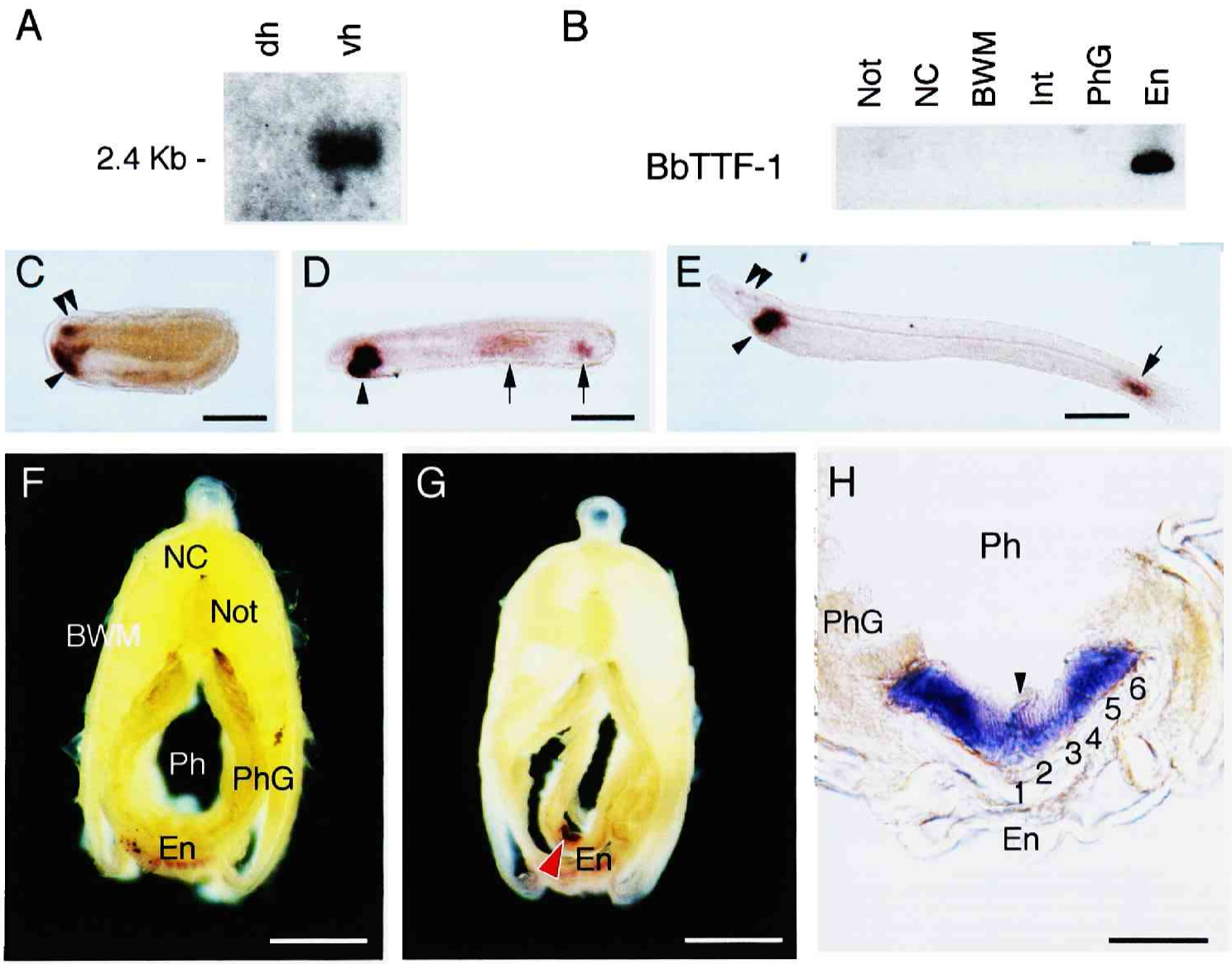
Mouse Nkx-2.3
88
Human Nkx-2.5
98
Mouse Nkx-2.5
89
CiTTF-1
90
HrTTF-1
80
BbTTF-1
AmphiNk2-1
Dog Nkx-2.1
98
Human Nkx-2.1
Mouse Nkx-2.1
78
Chick Nkx-2.1
C.elegans CEH-22
91
Mouse Nkx-2.2
0.1
Drosophila NK2
AmphiNk2-2

ıre 4


A
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BbTTF-1
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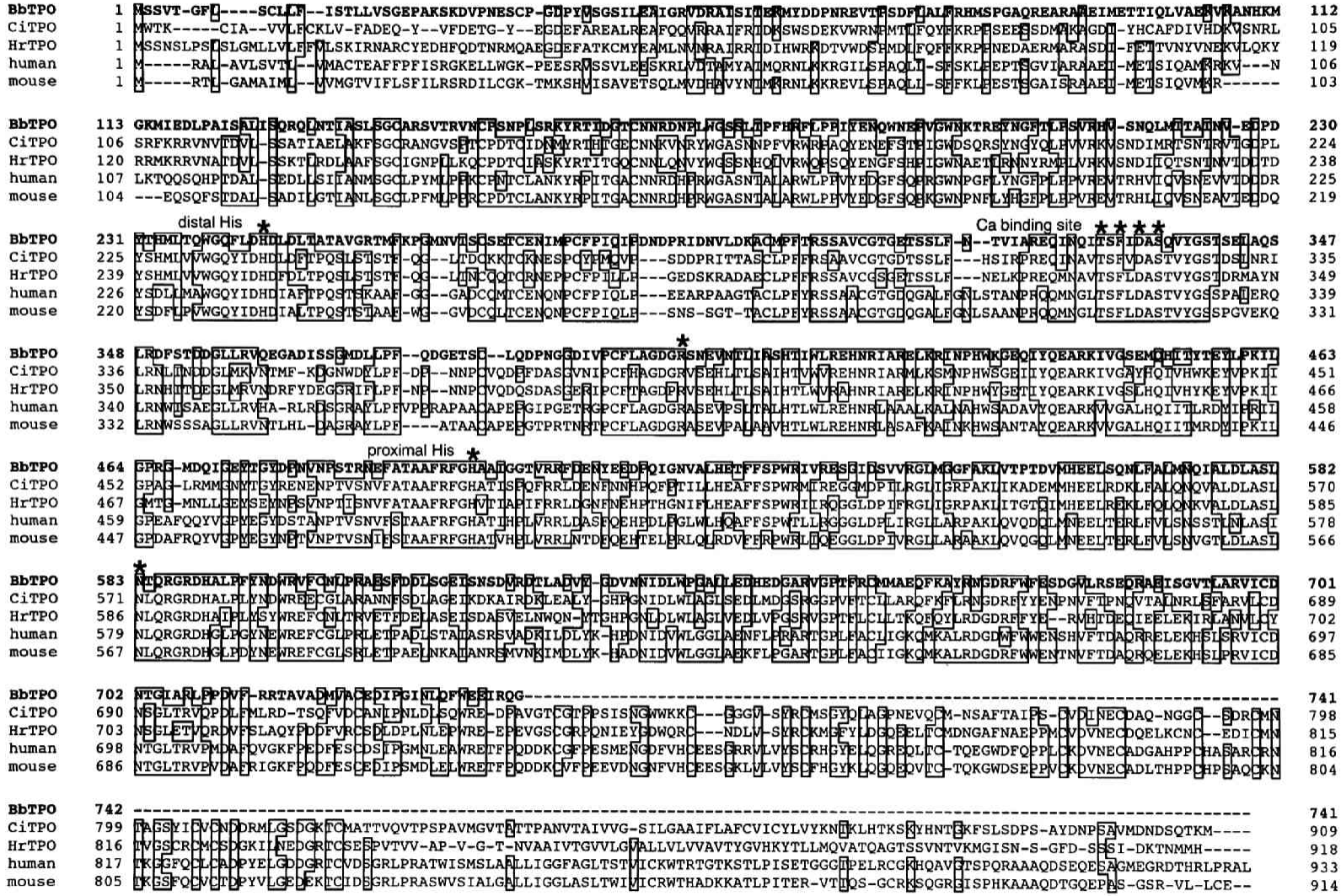
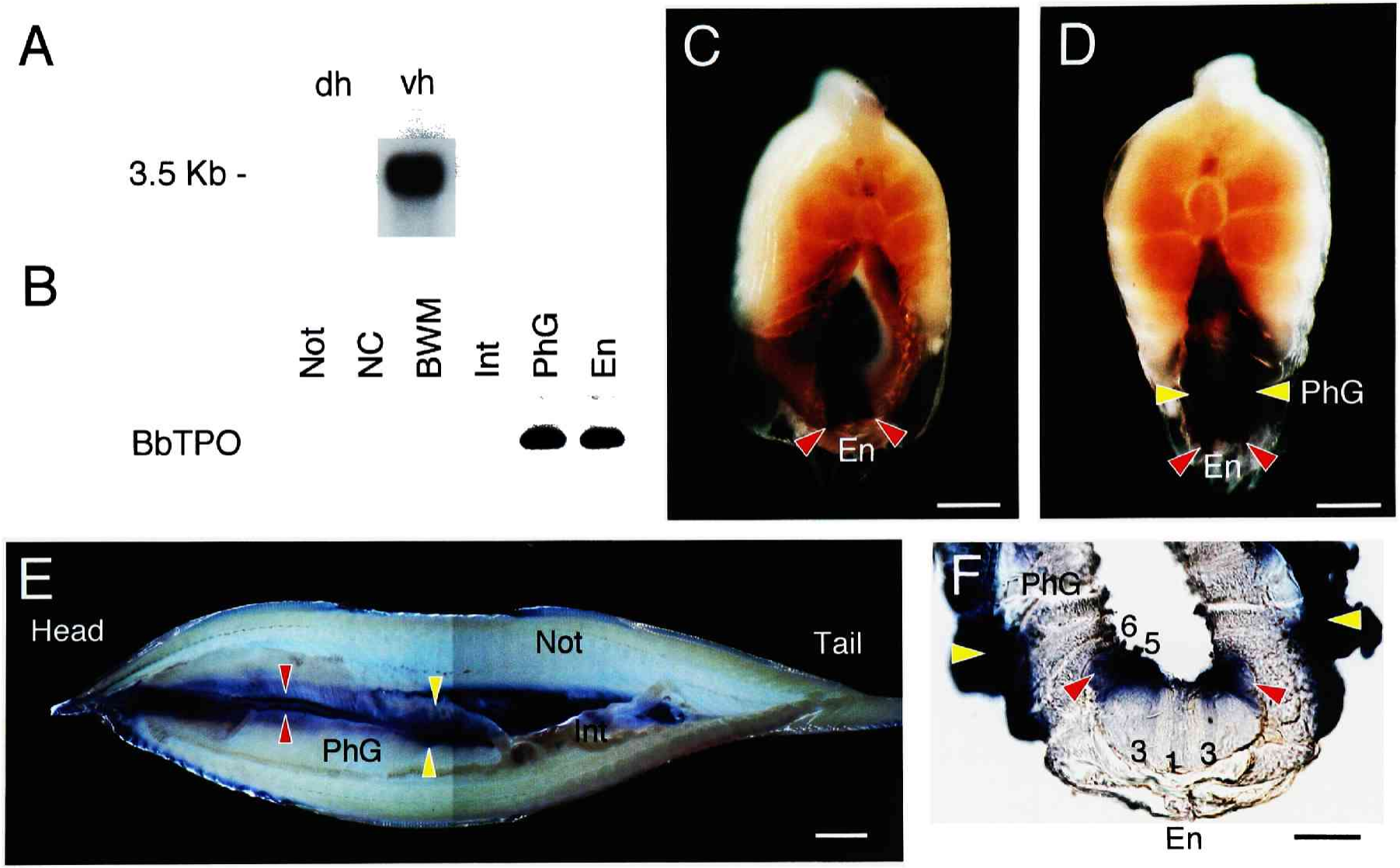
ıre 5



re 6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3.5 Kb -
B
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NC
BWM
Int
PhG
En
BbTPO
C
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PhG
En
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Head
Not
Tail
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Int
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PhG
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ORIGINAL ARTICLE

Michio Ogasawara

Overlapping expression of amphioxus homologs of the thyroid transcription factor-1 gene and thyroid peroxidase gene in the endostyle: insight into evolution of the thyroid gland

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Abstract The endostyle is a pharyngeal organ of urochordates, cephalochordates, and primitive vertebrates. This organ has iodine-concentrating and iodine-metabolism activities, and therefore the endostyle is considered to be homologous to the follicle of the thyroid gland. In higher vertebrates the genes for both thyroid transcription factor-1 (TTF-1) and thyroid peroxidase (TPO) are expressed in the thyroid gland follicle. TTF-1 regulates the expression of *TPO*, which encodes an iodinating enzyme associated with thyroid hormone synthesis. A recent study showed that the ascidian *TTF-1* and *TPO* genes are specifically expressed in the endostyle, but that the expression domains of these genes are not overlapping, suggesting that ascidian *TPO* is not regulated by TTF-1. To investigate the molecular mechanisms involved in the formation and function of the endostyle, with special reference to the evolution of the follicle of the thyroid gland, I isolated and characterized cDNA clones for the amphioxus homologs of the *TTF-1* gene (*BbTTF-1*) and *TPO* gene (*BbTPO*) from *Branchiostoma belcheri*. Reverse transcriptase–polymerase chain reaction/Southern blotting revealed that both amphioxus *TTF-1* and *TPO* genes are expressed mainly in the adult endostyle. Spatial and temporal expression patterns assessed by in situ hybridization revealed that *BbTTF-1* is expressed in the endodermal cells during early embryogenesis and is maintained in all zones of the adult endostyle. On the other hand, expression of *BbTPO* is chiefly in zones 5 and 6 of the adult endostyle where it overlaps with that of *BbTTF-1*, and to a lesser extent in zones 1 and 3. This restriction of the expression of *BbTTF-1* and *BbTPO* to the endostyle strongly suggests that the endostyle is homologous to the follicle of the thyroid gland. Moreover, the spatial and temporal expression patterns of these genes suggest that *TTF-1* regulates *TPO* expression. The coexpression of these genes in amphioxus suggests that regulation of *TPO* by TTF-1 was present in the common ancestor of cephalochordates (acraniates) and craniates.

Key words Amphioxus · Thyroid transcription factor-1 · Thyroid peroxidase · Endostyle · Thyroid gland

Edited by N. Satoh

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Introduction

The endostyle is located in the ventral part of the pharynx of urochordates (tunicates), cephalochordates (acraniates), and ammocoetes (larval lampreys). In addition to the notochord, dorsal hollow neural tube, and pharyngeal gill slit, the endostyle is therefore a key structure for understanding the origin and evolution of the chordate body plan (e.g., Brusca and Brusca 1990; Gee 1996; Nielsen 1995; Willmer 1990). The chordate endostyle is thought to have two major functions. One is in protein secretion for catching food particles from sea water, i.e., for filter feeding. Ultrastructural studies of the endostyle have demonstrated that the endostyle contains several types of glandular cells, which occupy a large part of the endostyle (Barrington 1958; Fujita and Honma 1968; Fujita and Nanba 1971). Previous molecular studies of ascidians endostyle indicated that large amounts of transcripts which encode the predicted secretory proteins are expressed in zones of the protein-secreting elements (Ogasawara and Satoh 1998; Ogasawara et al. 1996). The other function of the endostyle appears to be equivalent to that of the vertebrate thyroid gland follicle. Histochemical studies in the ascidian (Dunn 1974; Fujita and Sawano 1979; Thorpe et al. 1972), amphioxus (Tsuneki et al. 1983), and larval lamprey (Fujita and Honma 1968) revealed iodine concentrating and thyroid peroxidase activity in the endostyle (reviewed by Eales 1997). Because of these thyroidlike properties and because the lamprey endostyle transforms into the follicle of the thyroid gland during the metamorphosis (Wright and

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Youson 1976), the endostyle is generally accepted to be homologous to the vertebrate follicle thyroid gland and to be its evolutionary precursor (e.g., Barrington 1957; Dunn 1974, 1980; Fujita and Sawano 1979; Salvatore 1969; Thorpe et al. 1972).

In the adult amphioxus the endostyle forms a groove in the floor of the pharynx and extends from the forepart of the pharynx to the esophagus (see Fig. 6E). Morphological observations of the amphioxus endostyle demonstrated that this organ forms a trough-shaped structure similar to the ascidian endostyle, and that the organ is divided into six different zones that run parallel to one another in longitudinal orientation (Barrington 1958). Ultrastructural and histochemical studies of the amphioxus endostyle revealed histochemical activities of peroxidase (Fredriksson et al. 1985; Tsuneki et al. 1983) and iodine-concentrating activity (Dunn 1974; Ericson et al. 1985; Fredriksson et al. 1984; Fujita and Honma 1969; Thorpe et al. 1972) in the cells of zones 5 and 6, and therefore these zones are considered to be the thyroid-equivalent region.

The thyroid gland follicle of higher vertebrates concentrates iodine and synthesizes thyroid hormones. Several thyroid-specific molecules have been isolated which are essential for thyroid hormone synthesis. These include thyroglobulin (Tg), an iodinated protein (Caturegli et al. 1997; Di Lauro et al. 1985; Malthiery and Lissitzky 1987; Mercken et al. 1985; van Ommen et al. 1989), and thyroid peroxidase (TPO), which iodinates Tg (Fujita and Sawano 1979; Isozaki et al. 1989; Kimura et al. 1989; Kotani et al. 1993; Magnusson et al. 1987; Taurog 1974). Both occur in the differentiated thyroid follicle and are therefore useful markers of the vertebrate thyroid gland follicle. In addition, several transcription factors have been identified (Lazzaro et al. 1991; Plachov et al. 1990; Zannini et al. 1997), including thyroid transcription factor-1 (*TTF-1*; also known as *Nkx-2.1*, *Titf1*, and *T/ebp*) which encodes a protein containing an NK-2 type homeodomain. Vertebrate *TTF-1* is expressed in the follicular thyroid gland, in the lung, and in the anterior nerve cord (Civitareale et al. 1993; Kimura et al. 1996; Lazzaro et al. 1991; Pera and Kessel 1998). In the follicular thyroid gland, TTF-1 directly regulates expression of *Tg*, *TPO*, and the thyrotropin receptor gene (Civitareale et al. 1993; Damante et al. 1994; Mascia et al. 1997). The *TTF-1* gene has also been isolated from amphioxus, where it is expressed in the larval endostyle and other tissues; however, expression in adults has not been determined (Venkatesh et al. 1999).

We have isolated and characterized several endostyle-specific genes of ascidians which are expressed in the protein-secreting zones and encode novel secretory proteins presumably used for trapping food (Ogasawara and Satoh 1998; Ogasawara et al. 1996). In addition, we have cloned ascidian homologs of *TTF-1* and *TPO* which are expressed in nonoverlapping patterns in the endostyle (Ogasawara et al. 1999b). Therefore it does not appear that the ascidian TTF-1 direct regulates the expression of *TPO* in the adult endostyle as it does in vertebrates. In the present study, to determine whether TTF-1 regulates *TPO* gene in the amphioxus endostyle, I isolated and characterized the homologs of amphioxus *TTF-1* and *TPO* from *Branchiostoma belcheri*. Both genes are expressed in the adult endostyle, in partially overlapping patterns. Therefore the present study provides the first evidence of overlapping expression of *TTF-1* and *TPO* in the endostyle of lower chordates.

Materials and methods

Biological materials

Specimens of *B. belcheri* were collected during the spawning season at Ariake-Kai near the Aizu Marine Biological Station of Kumamoto University, Kyushu, Japan, and at Gokasyo-bay near the National Research Institute of Aquaculture, Mie, Japan. Naturally spawned eggs were fertilized and raised at room temperature. They developed into gastrulae, neurulae, and larvae, about 5, 10, and 48 h after fertilization, respectively. Samples at appropriate developmental stages were fixed for in situ hybridization. Adult specimens were sectioned transversely at about 3-mm intervals and immediately fixed for in situ hybridization. These specimens were kept at –20°C until use. Adult specimens and dissected endostyles were frozen in liquid nitrogen for RNA isolation and kept at –80°C until use.

Isolation and sequencing of cDNA clones for amphioxus TTF-1 and TPO genes

The sense-strand oligonucleotide primer for TTF-1, NKX-F1: 5'-TT (CT)AG (CT)CA (AG)GCNCA (AG)GTNTA (CT)GA (AG)(CT)T-3', which corresponds to the amino acid sequence FSQAQVYEL, and the antisense oligonucleotide primer NKX-R: 5'-(GT)TT (CT)TG (AG)AACCA (AGT)AT (CT)TTNAC (CT)TG-3', which corresponds to the amino acid sequence QVKIWFQN, were synthesized based on conserved NK-2 type homeodomains from *Caenorhabditis elegans*, *Drosophila*, tunicates, amphioxus, and vertebrates. Target fragments were amplified by reverse transcriptase (RT) polymerase chain reaction (PCR) from adult endostyle total RNA and 131-bp PCR product was obtained. Oligonucleotide primers for TPO, TPO-F: ACIGCIGCITT (TC)(CA)GITT (TC)GGICA, which corresponds to the amino acid sequence TAAFRFGH, and the antisense oligonucleotide TPO-R: GGIA (AG)ICC (AG)TG (AG)TCIC (GT)ICCIC (GT)(TC)TG, which corresponds to the amino acid sequence QRGRDHGLP, were synthesized based on the conserved region of the TPO. Target fragments were amplified by RT-PCR from adult endostyle total RNA and 308-bp PCR product was obtained. These PCR fragments were randomly labeled with [^{32}P]dCTP (Amersham), and 3.0×10^5 phages of a mixed cDNA library of *B. belcheri* adult and gastrula (Terazawa and Satoh 1997) were screened under hybridization condition of 6× SSPE, 0.1% SDS, 1× Denhardt's solution, 50% formamide at 42°C for 16 h, and washing condition of 2× SSC, 0.1% SDS at 55°C for 30 min, 1× SSC, 0.1% SDS at 55°C for 30 min, 0.1× SSC, 0.1% SDS at 55°C for 30 min. Isolated clones were sequenced using an ABI PRISM 377 DNA Sequencer (Perkin Elmer).

Sequence comparisons and molecular phylogenetic analyses

Sequences were aligned using the SeqQpp 1.9 manual aligner for Macintosh (Gilbert 1993). Phylogenetic analyses were performed on the amino acid sequences of the homeodomain. Estimation of molecular phylogeny was carried out by the neighbor-joining method (Saitou and Nei 1987) using the CLUSTAL V (Higgins et

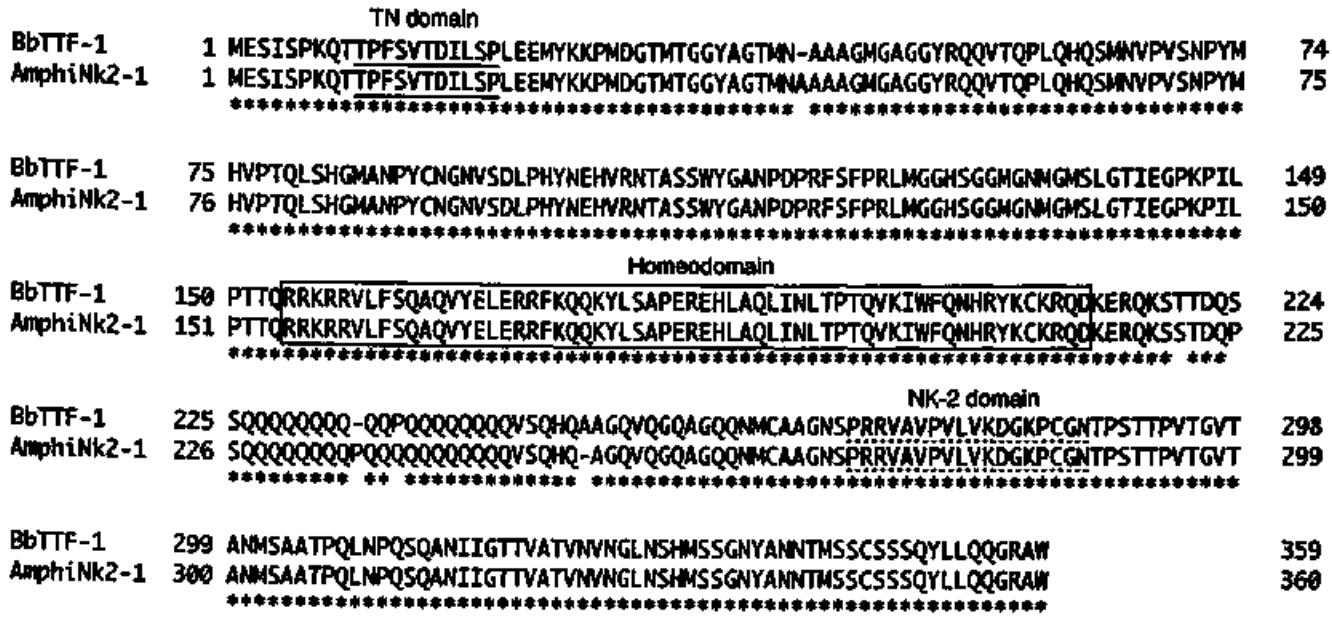


Fig. 1 Predicted amino acid sequences of thyroid transcription factor-1 gene product (*BbTTF-1*) of *Branchiostoma belcheri* (*above*), and sequence comparison with AmphiNk2-1 of *B. floridae* (*below*; Venkatesh et al. 1999). The polypeptide encoded by BbTTF-1 contains 359 amino acids, and the molecular mass is estimated to be 39.5 kDa. The nucleotide sequence of *BbTTF-1* will appear under the DDBJ/EMBL/GenBank accession no. AB028842. *Asterisks* Amino acids identical between BbTTF-1 and AmphiNk2–1 (98.3%). The TN domain (*underlined*), NK-2-type homeodomain (*boxed*), and NK-2 domain (*dotted underlined*) are identical in BbTTF-1 and AmphiNk2-1

al. 1992) program. Confidence in the phylogeny was assessed by bootstrap resampling of the data (×1000; Felsenstein 1985).

Northern blot analysis

Total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987), and poly(A)$^+$ RNA was purified with Oligotex dT30 beads. Northern blot hybridization was carried out using standard procedures (Sambrook et al. 1989), and filters were washed under high-stringency conditions (hybridization: 6× SSPE, 0.1% SDS, 1× Denhardt's solution, 50% formamide at 42°C for 16 h; washing: 2× SSC-0.1% SDS at 60°C for 30 min, 1× SSC-0.1% SDS at 60°C for 30 min, 0.1× SSC-0.1% SDS at 60°C for 30 min). The entire region of cDNAs was labeled with [^{32}P]dCTP using a random primed labeling kit (Boehringer-Mannheim) for hybridization probes.

RT-PCR/Southern blot analysis

Total RNA of various tissues (endostyle, gill, intestine, body-wall muscle, notochord, and nerve cord) was extracted from small pieces of adult tissues that were sonicated in an extraction buffer containing guanidinium-thiocyanate. Total RNA was isolated from the extracts by protease K and phenol-chloroform treatment. Total RNA (10 μl) was reverse-transcribed after hybridization with oligo dT primer, and then PCR (30 cycles: 1 min at 94°C, 2 min at 50°C and 1 min at 72°C) was carried out with specific primers. Primers for *BbTTF-1* are BbTTF-1F; 5'-CTAGTCAAAGACGGCAAGCCG-3' and BbTTF-1R; 5'-TGCTACAATACTGGCACGTCC-3', and for *BbTPO* (*B. belcheri* thyroid peroxidase) are BbTPOF; 5'-GAGCAGTTCAAGGCATATCGC-3' and BbTPOR; 5'-CATAATGACGCTGTACCGTGC-3'. Amplified fragments were blotted onto Hybond-N+ nylon membranes (Amersham). The blots were hybridized and washed under the same conditions as for northern blotting.

In situ hybridization

Whole-mount in situ hybridization was carried out essentially as described by Holland et al. (1992). Embryos and transversely sectioned specimens of adults were fixed in 4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS buffer at 4°C for 12 h. Probes were synthesized by following the instructions from the supplier of the labeling kit (DIG RNA Labeling Kit; Boehringer-Mannheim). Some specimens were embedded in polyester wax (BDH Chemicals) and sectioned at 10-μm intervals for observation at high magnification.

Results

Isolation and characterization of cDNA clones for *TTF-1* (*Nkx-2.1*) gene of the amphioxus *B. belcheri*

The longest cDNA clone included 2353 nucleotides excluding the poly(A) tail and, as is evident from the northern blotting shown in Fig. 4A, this cDNA covered the full-length sequence of the gene transcript. This sequence contained a single open reading frame (ORF) that encoded a polypeptide of 359 amino acids, and the molecular mass (M_r) of the predicted protein was 39.5 kDa. This cDNA clone encoded a conserved TN domain, NK-2-type homeodomain, and NK2 domain (Figs. 1, 2), and therefore we named this gene *BbTTF-1* (*B. belcheri TTF-1*).

Figure 1 compares the amino acid sequence of BbTTF-1 to that of TTF-1 (AmphiNk2-1) from another species of *Branchiostoma*, *B. floridae* (Venkatesh et al. 1999). The amino acid sequences of BbTTF-1 and AmphiNk2-1 was very similar (98.3%), and TN domains (underlined), NK-2-type homeodomain (boxed), and

A

	TN domain	
BbTTF-1	TPFSVTDILSP	(100.0)
AmphiNk2-1	.....	(100.0)
HrTTF-1	.....	(100.0)
CiTTF-1	.....	(100.0)
Human TTF-1(Nkx-2.1)	.....S.....	(90.9)
Mouse TTF-1(Nkx-2.1)	.....S.....	(90.9)
AmphiNk2-2	.S.T.K...DM	(54.5)
Mouse Nkx-2.2	.G...K...DL	(63.6)
Mouse Nkx-2.3	.....K...NL	(72.7)
Mouse Nkx-2.5	.....K...NL	(72.7)

B

	Homeodomain ↓	
BbTTF-1	RRKRRVLFSQAQVYELERRFKQQKYLSAPEREHLAQLINLTPTQVKIWFQNHRYKCKRQD	
AmphiNk2-1	.....	(100.0)
HrTTF-1	.....F.....M.R.....N..AL	(90.0)
CiTTF-1	.....F.....M.H.....N..SL	(90.0)
Human TTF-1(Nkx-2.1)	.....SM.H.....M...A	(91.7)
Mouse TTF-1(Nkx-2.1)	.....SM.H.....M...A	(91.7)
AmphiNk2-2	K .....K..T.....R..R.....R..R.....AQ	(85.0)
Mouse Nkx-2.2	K.....K..T.....R..R.....S..R.....M..AR	(83.3)
Mouse Nkx-2.3	...P.....F.....R.....SSLK..S.....R.....R	(83.3)
Mouse Nkx-2.5	.....R.....DQ..SVLK..S.....R.....R	(83.3)
Drosophila NK2	K.....TK..T.....R..R.....S..R.....T..AQ	(81.7)
Drosophila NK3	KKRS.AA..H...F.....A..R...G...SEM.KSLR..E.....R...T..KQ	(61.7)
Drosophila NK4	K..P.....L...C..RLK...TGA...II..KL..SA.....R...S..G.	(68.3)
Drosophila NK1	..RA.TA.TYE.LVS..NK..TTR...VC..LN..LSLS..E.....R.T.W.KQN	(50.0)

Fig. 2 Comparison of the amino acid sequences of the TN domain (A) and homeodomain (B). Human TTF-1 (Saiardi et al. 1995), mouse TTF-1 (Oguchi et al. 1995), amphiNk2-1 (Venkatesh et al. 1999), HrTTF-1 (Ogasawara et al. 1999a), CiTTF-1 (Ristoratore et al., submitted), amphiNk2-2 (Holland et al. 1999), mouse Nkx-2.2 (Price et al. 1992), mouse Nkx-2.3 (Price et al. 1992), mouse Nkx-2.5 (Lints et al. 1993), and *Drosophila* NK1-NK4 (Kim and Nirenberg 1989). *Right* Percentage identity; *dots* identical amino acids. All NK-2-type homeodomains (TTF-1/Nkx-2.1, Nkx-2.2, Nkx-2.3, Nkx-2.4, Nkx-2.5, NK2, NK3 and NK4) share a tyrosine residue at position 54 within the domain (*arrow*)

NK2 domain (dotted underlined) are identical. Furthermore, the nucleotide sequences of ORFs and 5' untranslated regions (UTRs) were highly conserved (93.9% and 98.9% identical, data not shown) between the genes, and the 3' UTR sequences also closely resemble each other (73.8% identical). The overall identity of the entire DNA sequence was 86.4%.

Sequence comparison and molecular relationships of TTF-1 proteins

Figure 2 shows that the TN domains, homeodomains, and NK-2 domains of the TTF-1 are highly conserved among the chordates (over 90% identity). On the other

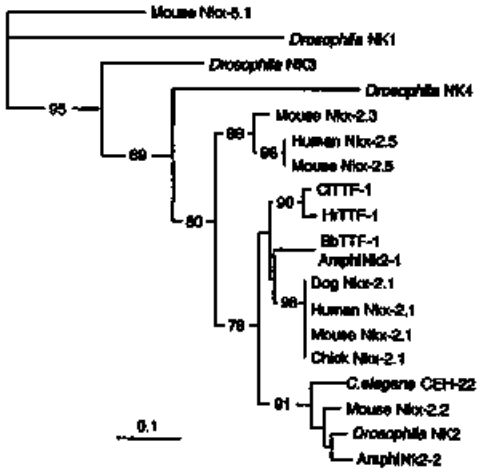


Fig. 3 Phylogenetic relationships among TTF-1 (Nkx-2.1) proteins. The tree was constructed by means of the neighbor-joining method using the amino acid sequences of the homeodomain. *Numbers at branches* bootstrap percentages (only those over 50% are shown)

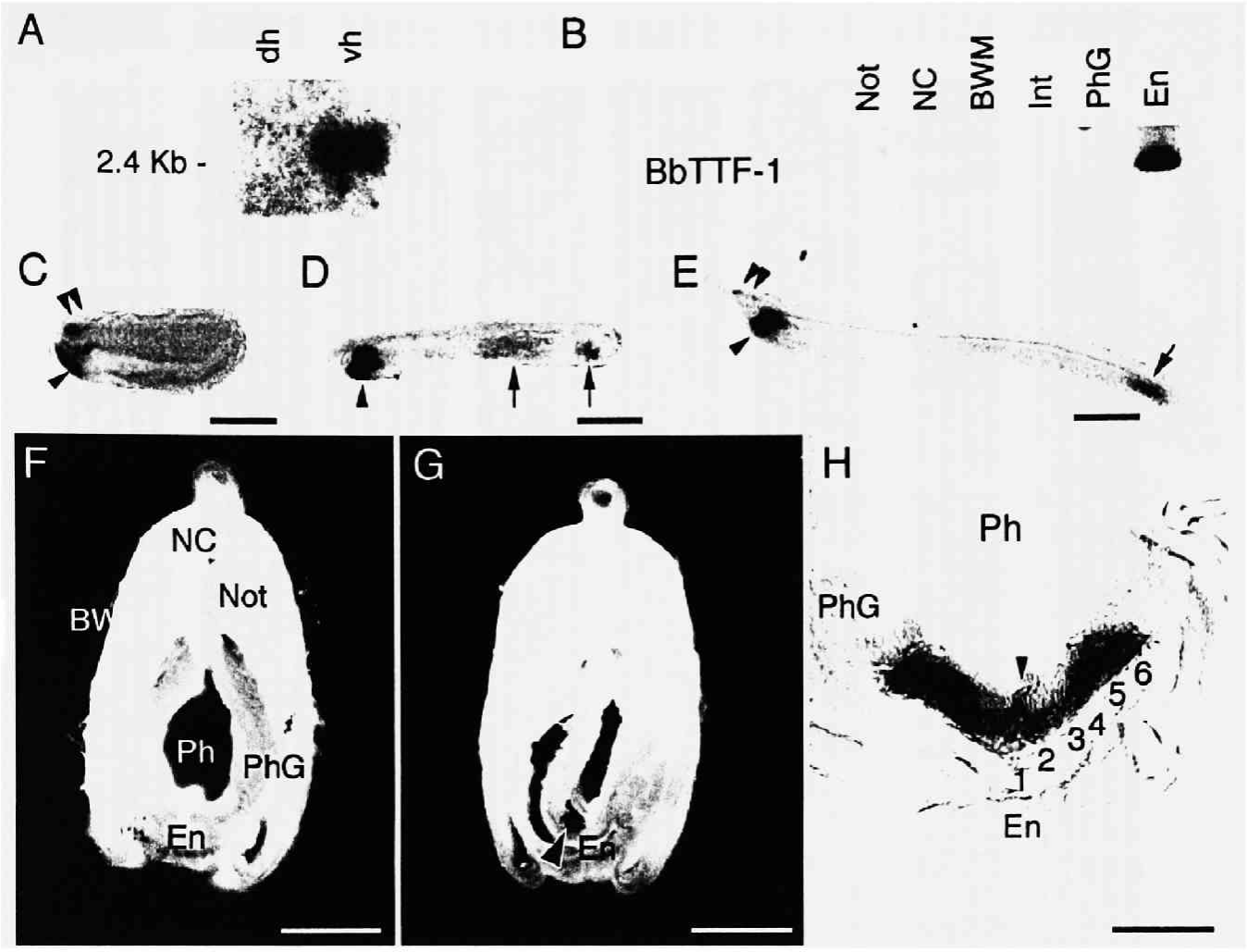


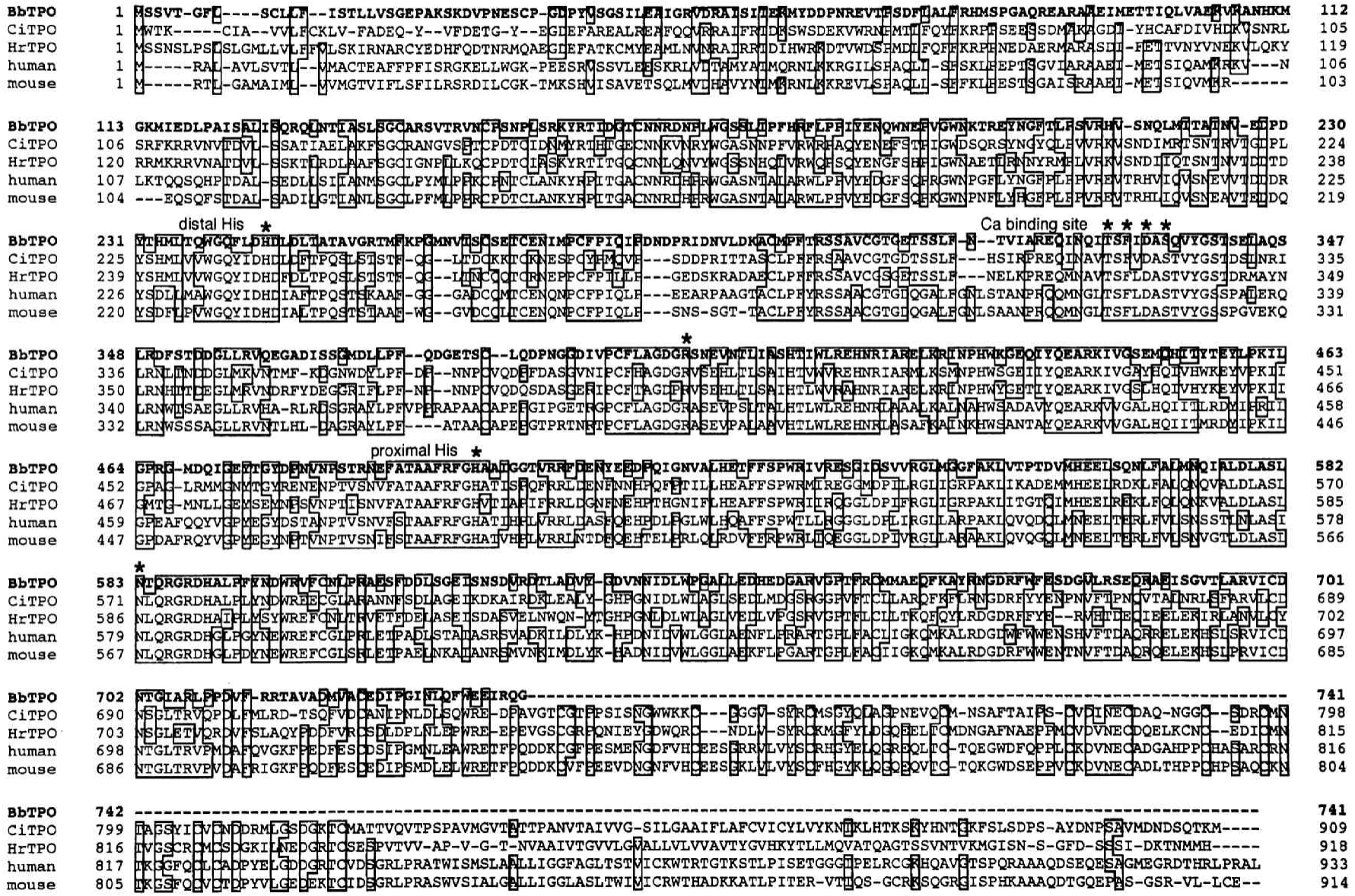
Fig. 4A–H Distribution of *BbTTF-1* transcript in *B. belcheri*. **A** Northern blots of 5 μg poly(A)$^+$ RNA prepared from the dorsal half (*dh*) and ventral half (*vh*) of adults. **B** RT-PCR/Southern blot analysis of *BbTTF-1* expression. Notochord (*Not*), nerve chord (*NC*), body-wall muscle (*BWM*), intestine (*Int*), pharyngeal gill (*PhG*), and endostyle (*En*). **C–E** Localization of *BbTTF-1* transcript in embryos and larvae as revealed by in situ hybridization. *Scale bars* 100 μm. **C** At the neurula stage, the transcript is detected in the endodermal region (*arrowhead*) and anterior part of the neural tube (*twin arrowheads*). **D** At the late neurula stage the transcript is detected in the pharynx (*arrowhead*) and middle and posterior part of the gut (*arrows*). **E** In larva, transcript is detected in the endostyle primordium (*arrowhead*) and hindgut (*arrow*). Weak expression in the anterior part of the neural tube (*twin arrowheads*) is also detected. **F–H** Expression of *BbTTF-1* in the adult amphioxus. Sectioned adult specimens are hybridized as whole-mount specimen with *BbTTF-1* sense probe as a negative control (**F**) and with antisense probe (**G**). No signals above background are detected in **F**. A distinct hybridization signal is detected only in the endostyle (*red arrowhead* in **G**). *Scale bars* **F**,**G** 1 mm. **H** The endostyle is sectioned transversely and observed at high magnification. *Scale bars* 100 μm. Signals for *BbTTF-1* expression are detected throughout the endostyle, with very strong signals in zones 5 and 6. In some specimens strong signals for *BbTTF-1* expression are also detected in zone 1 (*arrowhead*). *En* Endostyle; *PhG* pharyngeal gill; *Ph* pharynx; *Not* notochord; *BWM* body-wall muscle; *NC* nerve cord

hand, BbTTF-1 showed a lower level of amino acid sequence identity than other NK-2-type proteins (Nkx-2.2, Nkx-2.3 and Nkx-2.5).

The evolutionary relationships of the TTF-1 proteins were examined by molecular phylogenetic analyses (Fig. 3). A molecular phylogenetic tree was constructed by the neighbor-joining method based on the amino acid sequence of the homeobox domain. *Drosophila* NK1 was used as an outgroup. As is evident from this tree, amphioxus BbTTF-1 forms a group with Nkx-2.1 (mouse TTF-1/Nkx-2.1, human TTF-1/Nkx-2.1, dog Nkx-2.1, chick Nkx-2.1, amphioxus AmphiNK2–1, and ascidian CiTTF-1 and HrTTF-1). In addition, this group is included in a larger clade with *Drosophila* NK2, *C. elegans* CEH-22 (Okkema and Fire 1994), mouse Nkx-2.2 (Price et al. 1992), and amphioxus Nkx-2.2 (Holland et al. 1999). This larger clade was supported by a bootstrap value of 79%. From these data, together with the results mentioned above, we conclude that BbTTF-1 is a member of the Nkx-2.1 family.

BbTTF-1 is expressed in the endostyle

The expression of *BbTTF-1* was examined by northern blot analysis, RT-PCR/Southern blot analysis and in situ


distal His
Ca binding site
proximal His

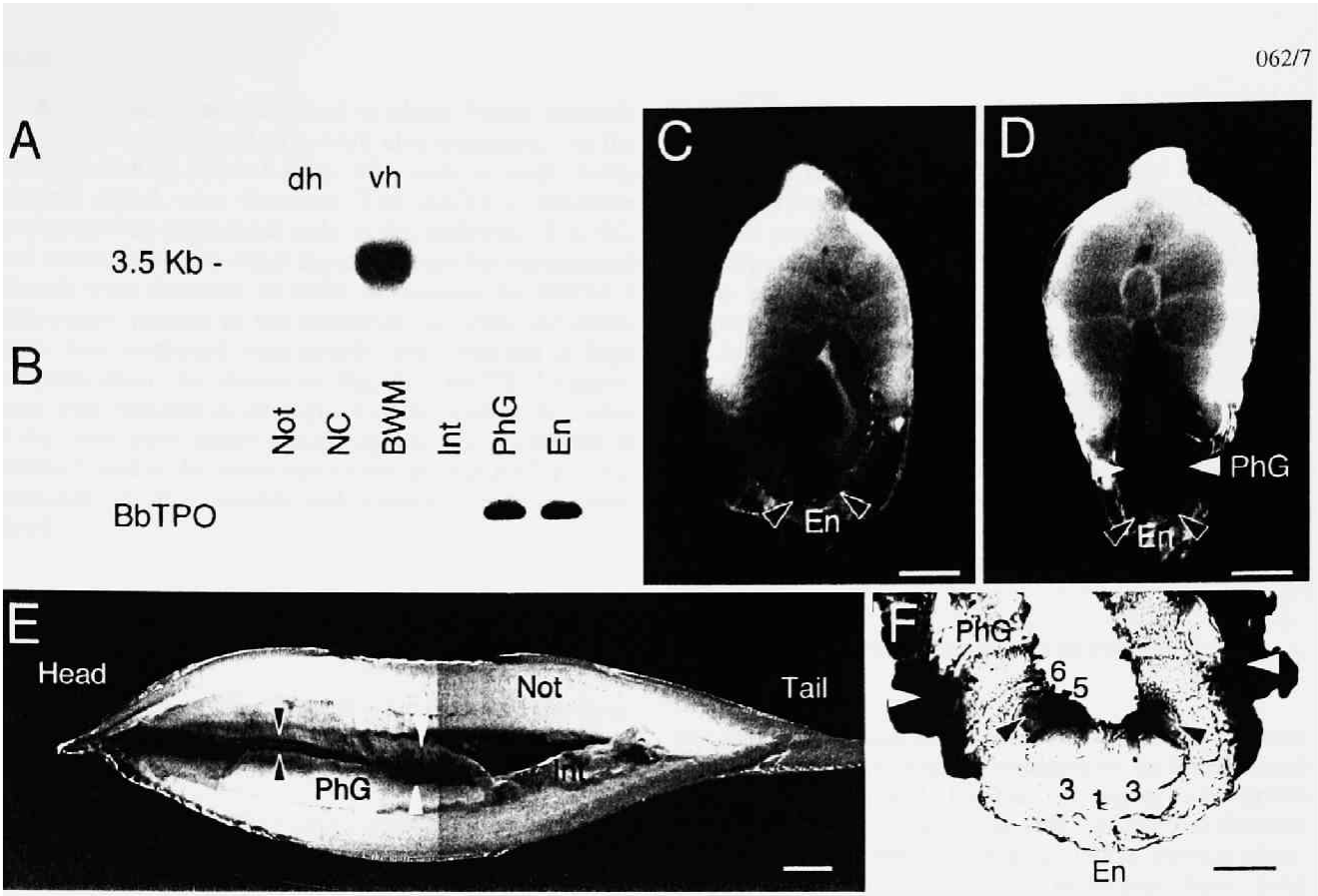


Fig. 6A–F Distribution of *BbTPO* transcript in *B. belcheri*. **A** Northern blots of poly(A)$^+$ RNA prepared from the dorsal half (*dh*) and ventral half (*vh*) of adults. **B** RT-PCR/Southern blot analysis of *BbTPO* expression. **C–F** Expression of *BbTPO* in the adult amphioxus revealed by in situ hybridization. **C,D** Sectioned adult whole-mount specimens are hybridized with *BbTPO* antisense probe. *Scale bars* 1 mm. Two stripes of hybridization signals are detected in the endostyle (*red arrowheads*). In some specimens hybridization signals were also detected in the outer wall of the pharyngeal gill bars (**D**, *yellow arrowheads*). **E** Adult specimens are dissected partially from the dorsal side and viewed from a dorsal position. Two stripes of *BbTPO* signal are detected in the endostyle (*red arrowheads*), and also in the posterior part of the pharyngeal gill (*yellow arrowheads*). *Scale bars* 1 mm. **F** The endostyle is sectioned transversely and observed at high magnification. *Scale bar* 100 μm. The *BbTPO* signal is detected in zones 5 and 6 (*red arrowheads*), and also detected in the outer wall of the pharyngeal gill (*yellow arrowheads*). A very weak signal is detected in zones 1 and 3. *En* Endostyle; *PhG* pharyngeal gill; *Int* intestine; *Not* notochord

Fig. 5 Comparison of the amino acid sequences of TPO proteins. BbTPO encoded a protein of 741 amino acids and calculated molecular mass (M_r) was 82.7 kDa. Nucleotide and predicted amino acid sequences of *BbTPO* will appear under the DDBJ/EMBL/GenBank accession no. AB028841. Amino acids identical with those of CiTPO (Ogasawara et al. 1999b), HrTPO (Ogasawara et al. 1999b), human TPO (Kimura et al. 1989), and mouse TPO (Kotani et al. 1993) are boxed. The overall identity between the amino acid sequences of BbTPO and the TPO-type peroxidases are: human TPO, about 43.4%, mouse, TPO, about 41.7%, CiTPO, about 46.9%, and HrTPO, about 46.1%; these identities are higher than those between BbTPO and non-TPO type peroxidase [human salivary peroxidase (38.3%), human eosinophil peroxidase (38.8%), human myeloperoxidase (40.7%), and sea urchin ovoperoxidase (33.8%)]. BbTPO lacks a carboxy-terminal tail. All amino acids required for peroxidase activity are completely conserved (*asterisks*)

hybridization. Northern blots using poly(A)$^+$ RNA prepared from the ventral and dorsal halves of adults detect a single band of *BbTTF-1* transcript of about 2.4 kb only in the ventral half of the adult (Fig. 4A). The length of the transcript roughly coincided with that of the longest cDNA characterized. Spatial expression of *BbTTF-1* in the adult tissues was also examined by RT-PCR/Southern blot analysis. The amplified product of the *BbTTF-1* transcript was detected only in the endostyle, and there was no signal in gill, intestine, muscle, notochord, of nerve cord (Fig. 4B). On the other hand, transcripts of the *B. belcheri* cytoplasmic actin gene were detected in all tissues, including notochord, nerve cord, body-wall muscle, intestine, pharyngeal gill, and endostyle (data not shown). Therefore transcripts of *BbTTF-1* were restricted to the endostyle of adult amphioxus.

In situ hybridization shows that expression of *BbTTF-1* in embryos and early larvae resembles that previously described for another amphioxus *TTF-1* gene, *AmphiNk2-1*. *BbTTF-1* expression at the neurula stage (Fig. 4C) is in the anterior endoderm (arrowhead) and the anterior part of the neural tube (twin arrowheads). In the late neurula (Fig. 4D), *BbTTF-1* was expressed mainly on the right side of the pharynx in the developing endostyle (arrowhead) and weak expression was observed in the middle and posterior parts of the gut (arrows). At the larval stage (Fig. 4E) *BbTTF-1* was expressed mainly in the endostyle primordium (arrowhead). Very weak expression was also observed in the anterior part of the neural tube (twin arrowheads) and in the hindgut (arrow).

Adult specimens sectioned at about 3-mm intervals across the longitudinal axis were also examined. For the sense probe as control (Fig. 4F), only a weak background signal was detected. The *BbTTF-1* antisense probe showed expression only in the endostyle (Fig. 4G, red arrowhead). No other signals except for background signals were detected. In order to examine the *BbTTF-1* expression pattern in the endostyle in detail the endostyle was sectioned transversely and observed at high magnification. As shown in Fig. 4H, *BbTTF-1* expression was detected in all regions of the endostyle (zones 1–6), with particularly strong signals being detected in zones 5 and 6. In some specimens strong *BbTTF-1* expression was also detected additionally in zone 1 (arrowhead).

Isolation and characterization of cDNA clone for amphioxus *TPO* gene

Eight cDNA clones for *BbTPO* were isolated. The longest contained 3449 nucleotides, excluding the poly(A) tail with an ORF that encoded a polypeptide of 741 amino acids.

cDNA clones of TPO genes have been isolated from several vertebrates and ascidians as well as amphioxus: human (Kimura et al. 1989), mouse (Kotani et al. 1993), and the ascidians *Ciona intestinalis* and *Halocynthia roretzi* (Ogasawara et al. 1999b). Overall the amino acid identities of BbTPO and TPO proteins of other chordates are between 41.7% and 46.9%. On the other hand, BbTPO shows less identity than non-thyroid-type peroxidase: its identity with human salivary peroxidase is 38.3% (Kiser et al. 1996), with human eosinophil peroxidase 38.8% (Ten et al. 1989), with human myeloperoxidase 40.7% (Hashinaka et al. 1988), and with sea urchin ovoperoxidase 32.9% (LaFleur et al. 1998; data not shown).

While all of the amino acids required for peroxidase activity (asterisks in Fig. 5) are completely conserved, BbTPO lacks the hydrophobic region of the carboxy-terminus, as in the case of the non-thyroid-type peroxidases, including salivary peroxidase, eosinophil peroxidase, myeloperoxidase, and ovoperoxidase (data not shown).

Expression of the amphioxus *BbTPO* gene in the endostyle

Northern blot analysis revealed the presence of a single *BbTPO* transcript of 3.5 kb (Fig. 6A) in the ventral half but not the dorsal half of adults. RT-PCR/Southern blot analysis of adult tissues showed *BbTPO* transcripts in the endostyle and pharyngeal gills only (Fig. 6B).

Whole-mount in situ hybridization did not detect *BbTPO* expression in embryos or larvae (data not shown). In adults *BbTPO* expression was limited to the endostyle (Fig. 6C, red arrowheads). In some specimens *BbTPO* expression was also detected in the outer wall of the pharyngeal gills (Fig. 6D, yellow arrowheads). To further clarify the latter expression pattern of *BbTPO*, adult amphioxus were partially dissected from the dorsal side and prepared as whole-mounts for in situ hybridization. Figure 6E shows two stripes of *BbTPO* expression in the endostyle (red arrowheads) extending from the forepart of the pharynx to the intestine. *BbTPO* expression in the pharyngeal gills was also detected in the posterior part of the pharynx near the intestine (yellow arrowheads). Sections show the *BbTPO* expression mainly in zones 5 and 6 (Fig. 6F, red arrowheads). In some specimens *BbTPO* expression was detected in the outer wall of the gill bars (yellow arrowheads) and in zones 1 and 3.

Discussion

TTF-1 and *TPO* are key molecules in evolution of the thyroid

The endostyle, which concentrates iodine and has peroxidase activity, is generally considered to be homologous to the thyroid gland follicle of higher vertebrates (Barrington 1957; Dunn 1974, 1980; Fujita and Sawano 1979; Salvatore 1969; Thorpe et al. 1972). Several mammalian thyroid follicle-specific molecules have been characterized, and thyroid-specific gene expression as well as gene regulation mechanisms have been analyzed in relation to thyroid development and function (Civitareale et al. 1989; Lazzaro et al. 1991; Plachov et al. 1990; Zannini et al. 1997).

One of these molecules, *TTF-1*, encodes a transcription factor with an NK-2-type homeodomain for DNA binding and is member of the *Nkx-2.1* gene family (Harvey 1996). In the mammalian thyroid follicle TTF-1 binds directly to the sequences upstream of the *TPO*, *Tg*, and thyrotropin receptor genes and regulates their expression (Civitareale et al. 1993; Damante et al. 1994; Mascia et al. 1997). Homologs of vertebrates *Nkx-2.1* have been isolated from two ascidian species, *C. intestinalis* (Ristoratore et al., submitted) and *H. roretzi* (Ogasawara et al. 1999a) and from amphioxus *B. floridae* (*AmphiNK2-1*; Venkatesh et al. 1999). In the present study a homolog (*BbTTF-1*) was isolated from another amphioxus *B. belcheri*. *BbTTF-1* encodes a typical NK-2-type homeodomain, and phylogenetic analysis indicates that *BbTTF-1* is a member of the *Nkx-2.1* gene family. The amino acid sequence of BbTTF-1 is highly conserved (98.3% identical) compared with that of AmphiNk2-1. The nucleotide sequences of the two genes also closely resemble each other.

The embryonic and larval expression pattern of *BbTTF-1* is identical to that of *AmphiNk2-1*. During embryogenesis both genes are expressed in embryonic gut, nerve cord, and endostyle (Venkatesh et al. 1999). RT-PCR/Southern blotting and in situ hybridization revealed that *BbTTF-1* expression in adults is restricted to the endostyle. These results suggest that amphioxus *TTF-1* is

associated with endostyle development and function. Thus *TTF-1* is appears to be a key molecule for understanding the development of the thyroid gland follicle and the endostyle.

On the other hand, *TPO* encodes an enzyme involved in iodinating Tg and is therefore important for thyroid hormone synthesis. This gene is considered to be a specific marker for differentiation of the thyroid gland follicle (Francis-Lang et al. 1992). cDNA clones of *TPO* have been isolated from several mammals (humans, mice, rats, and pigs), and their structures and functions have been well characterized. *TPO* homologs have been isolated from ascidians (Ogasawara et al. 1999b) and amphioxus (*BbTPO*, in the present study). The amino acids which are associated with peroxidase activity (Taurog and Wall 1998) are completely conserved among the various known TPOs. Thus not only ascidian TPOs but also amphioxus TPO might have peroxidase activity. Interestingly, the amphioxus TPO has no long hydrophobic carboxy-terminal tail which is thought to be required for membrane binding. Other peroxidases, such as lactoperoxidase (Dull et al. 1990) and myeloperoxidase, also lack this hydrophobic tail. Therefore, during the evolution from common ancestor of ascidian and amphioxus to the amphioxus *B. belcheri*, this characteristic tail might have been lost. However, whether TPO of other amphioxus species have a hydrophobic tail should be examined in other amphioxus species.

The strong expression of *BbTPO* in the adult endostyle is restricted to zone 5, and weak expression is detected in zone 6. This expression pattern coincides with that of histochemical peroxidase activities reported by Tsuneki et al. (1983), Fredriksson et al. (1984) and Ericson et al. (1985). In some individuals very weak expression is also detected in zones 1 and 3, and strong expression in the outer wall of the pharyngeal gill. These conserved sequence and expression patterns of *BbTPO* in the adult endostyle support the idea that the endostyle is homologous to the vertebrate thyroid gland follicle. Therefore *TPO* should be a useful molecule for analyzing the origin and evolution of thyroid function in the endostyle.

In addition, the overlapping expression of *BbTTF-1* and *BbTPO* suggests the possibility that TTF-1 regulates the *TPO* expression in amphioxus. The amino acid sequences, gene expression patterns, and gene expression mechanisms of *TTF-1* and *TPO* are comparable between vertebrates and lower chordates. Therefore these molecules will be useful not only for analyzing their functions in the endostyle but also for analyzing the molecular mechanisms involved in evolution of the thyroid-related function.

Evolution of the mechanism of regulation of thyroid-related gene expression

Previous studies of ascidian *TTF-1*s (*CiTTF-1* and *HrTTF-1*) and *TPO*s (*CiTPO* and *HrTPO*) indicated that these genes are expressed specifically in the endostyle. Observation of specimens at high magnification by in situ hybridization revealed that ascidian *TTF-1*s are mainly expressed in zones 1, 3, and 5 of the supporting elements. On the other hand, the ascidian *TPO*s are expressed in zone 7, which is one of the thyroid-equivalent elements. Therefore the expression patterns of the *TTF-1* and *TPO* genes do not overlap in the ascidian endostyle, suggesting that ascidian TTF-1 does not directly regulate ascidian *TPO* expression in the endostyle.

The organization of the amphioxus endostyle resembles that of the ascidian endostyle. The amphioxus endostyle is divided into six zones. From studies of the histochemical activities of peroxidase (Fredriksson et al. 1984; Tsuneki et al. 1983) and iodine-concentrating activity (Dunn 1974; Fredriksson et al. 1984; Ericson et al. 1985; Thorpe et al. 1972), the thyroid-equivalent regions are thought to be zones 5 and 6. The present study demonstrates that both amphioxus *TTF-1* and *TPO* are expressed in the endostyle, and that the expression domains overlap in zones 5 and 6. This result provides the first molecular evidence for overlapping expression of *TTF-1* and *TPO* in lower chordates, suggesting that this overlapping expression first appeared during the evolution to cephalochordates. However, some of the *BbTPO* expression domains do not coincide with the *BbTTF-1* expression domains. Especially the *BbTPO* expression domain in the outer wall of the pharyngeal gills does not overlap with the *BbTTF-1* expression domain. Therefore in the pharyngeal gills *BbTPO* expression might not be regulated by BbTTF-1. Furthermore, the occurrence of a *BbTTF-1* expression region in which there is no *BbTPO* expression suggests that BbTTF-1 is not an obligatory activator for *BbTPO*. Other molecules may be able to activate *BbTPO* gene expression. An alternative idea is that the expression domains of *BbTTF-1* and *BbTPO* are merely overlapping, and BbTTF-1 is not associated with regulation of the *BbTPO* expression. In order to analyze the regulation of *BbTPO* expression the upstream *cis*-regulatory sequences of *BbTPO* and possible gene-regulation-related interactions between TTF-1 and *TPO* should be examined. In any case, because *BbTTF-1* and *BbTPO* are expressed in the same zone of the amphioxus endostyle, possible gene regulation related interaction between TTF-1 and TPO should be examined.

Studies of the mammalian thyroid gland follicle have shown that several structural proteins and transcription factors are associated with the development and function of the thyroid gland. In mice, transcription factors TTF-1, TTF-2 and Pax8 bind to the upstream regulatory sequences of the *Tg*, *TPO*, and *thyrotropin receptor* genes to regulate their expression (Civitareale et al. 1989; Francis-Lang et al. 1992; Zannini et al. 1992, 1997). Recently amphioxus *B. floridae* homologs of *TTF-1* (*AmphiNk2-1*; Venkatesh et al. 1999) and *Pax8* (*AmphiPax2/5/8*; Kozmik et al. 1999) were isolated and characterized. The expression of these genes was detected in the developing and larval endostyle. Furthermore, an ascidian homolog of *Pax8* (*HrPax2/5/8*; Wada et al. 1998)

was also expressed in the adult endostyle (personal communication). Therefore these amphioxus and ascidian homologs might provide further knowledge for understanding the origin and evolution of the mechanism of gene regulation of thyroid-related molecules.

The lamprey is one of the most primitive vertebrates, and its larva also has a typical endostyle. This organ consists of several cell types, i.e., 1v, 1d, 2a, 2b, 2c, 3, 4, and 5 (Egeberg 1965; Fujita and Honma 1968). Histologically the organization differs from that of the endostyle of ascidians and amphioxus. However, iodine-concentrating activity was reported in cells of types 2c and 3 (Fujita and Honma 1969) and peroxidase activity in cells of types 2c and 3 (Tsuneki et al. 1983). These cells are located dorsolaterally to the glandular region as in the case of other endostyles. Therefore the types 2c and 3 cells are thought to be the thyroid-equivalent cells. Furthermore, transformation of the endostyle to the thyroid gland follicle during metamorphosis was confirmed histologically (Wright and Youson 1976). It will be very interesting to examine expression and mechanisms of gene regulation of *TTF-1* and *TPO* in the endostyle and thyroid gland follicle during lamprey metamorphosis.

Origin and evolution of the thyroid-related activities in the endostyle

To date, thyroid-related activities including iodine-concentrating activity and thyroid peroxidase activity, have been reported in various organisms: ascidians (sessile tunicates: Ascidiacea), amphioxus, larval lampreys, and also pelagic tunicates (Fredriksson et al. 1985) including *Oikopleura dioica* (Appendicularia), *Salpa fusiformis* (Desmomyaria), and *Dolioletta gegenbauri* (Cyclomyaria). The endostyle of all of these organisms contains large glandular regions and thyroid-related regions. However, the structures of the endostyles in these organisms vary depending on the organism. The number of cell types and shapes of the cells are not the same. Thyroid-related cells contain both iodine-concentrating activity and peroxidase activity and are uniformly located in a similar position (dorsolaterally to the glandular region). Therefore the position of the thyroid-related cells might have been established during the evolution of the urochordates. On the other hand, the thyroid-related functions of iodine concentration and iodoamino acid formation have been reported not only in chordates but also in echinoderms and hemichordates (reviewed by Eales 1997). Therefore iodine metabolism appeared at least by the time of evolution to chordates.

Acorn worms (hemichordates) are key organisms for analyzing the origin and evolution of the chordate body plan, because acorn worms are the most primitive deuterostomes, and they have remarkable gill slits. Acorn worms have no endostyle, but iodine-concentrating activity in the surface of the glandular cells and iodoamino acid has been reported (Gorbman et al. 1954). Molecular phylogenetic analysis using *TTF-1* and *TPO* should be performed in hemichordates and should provide helpful knowledge of the origin and evolution of the thyroid-related molecules and thyroid formation.

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